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PTO-1590 (1-2000)

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EMBO J 1992 April 11(4):1593-7

J. of Neurosci Res 1993 Feb 15, 34(3):371-6 *199 epitope

Neuroscience Letters 1993 April 16, 153(1):57-60

FEBS Letters 1995 Feb 20, 360(1):5-9

FEBS Letters 1994 March 14, 341(1):94-98

J. of Neurochem., 1995 April 64(4):1759-68

Neurochemistry International 1995 March 26(3):205-15

Biochem J., 1995 Aug 1, 309(Pt 3):941-9

Neurobiol of Aging 1996 March-April, 17(2):235-41

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Article title	The phosphatase inhibitor okadaic acid induces a phosphorylated paired helical filament tau epitope in human LA-N-5 neuroblastoma cells
Article identifier	030439409300162P
Authors	Vandermeeren_M Lubke_U Six_J Cras_P
Journal title	Neuroscience Letters
ISSN	0304-3940
Publisher	Elsevier Ireland
Year of publication	1993
Volume	153
Issue	1
Supplement	0
Page range	57-60
Number of pages	4
User name	Adonis
Cost centre	Development
PCC	$20.00
Date and time	Thursday, September 28, 2000 3:50:07 PM

Neuroscience Letters, 153 (1993) 57–60

NSL 09424

The phosphatase inhibitor okadaic acid induces a phosphorylated paired helical filament tau epitope in human LA-N-5 neuroblastoma cells

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(Received 26 November 1992; Revised version received 14 January 1993; Accepted 18 January 1993)

Key words: Alzheimer's disease; Phosphatase; Cytoskeleton; Neurofibrillary tangle; Microtubule-associated protein; Mitogen-activated protein kinase

Recently, a mitogen activated protein kinase has been implicated in the generation of a phosphorylated paired helical filament (PHF) epitope recognized by the monoclonal antibody AT8. This epitope consists of phosphorylated serines 199 and/or 202 of the human microtubule associated protein τ. Theoretically, aside from abnormal kinase activity, inhibition of phosphatase activity could also be involved in the abnormal phosphorylation status of the microtubule associated protein τ. To investigate this, we incubated LA-N-5 neuroblastoma cells with okadaic acid, a specific inhibitor of phosphatase 2A. We found that incubating neuroblastoma cells with okadaic acid induces the abnormally phosphorylated AT8 epitope. The effect of okadaic acid is time and dose dependent and is reversible. Our findings suggest that phosphatase activity is important in the regulation of the phosphorylation state of τ. Phosphatases may act directly on τ or may influence the activity of mitogen activated protein kinase. Incubation of LA-N-5 neuroblastoma cells with okadaic acid provides a cellular model in which the generation of a well-defined PHF-τ epitope can be investigated.

The neurofibrillary tangles of Alzheimer's disease (AD) are neuronal inclusions that consist of 20 nm paired helical filaments (PHF), predominantly composed of an abnormally phosphorylated microtubule associated protein τ [5]. The mechanism of this abnormal phosphorylation has not been completely elucidated, yet it is commonly assumed that an abnormal kinase activity is responsible for the aberrant phosphorylation of τ in AD [9]. Recently, a mitogen activated protein kinase (MAPkinase) has been described that can phosphorylate recombinant τ and thereby generate a phosphorylated PHF-epitope recognized by the monoclonal antibody AT8 [4, 7]. This AT8 epitope consists of phosphorylated serines 199 and/or 202 of human τ [2]. The complementary antibodies BT2 and tau-1 are directed to the same epitope, but their immunoreactivity is lost when Ser 199/202 are phosphorylated [3, 7].

The aberrant phosphorylation leading to the formation of the AT8 epitope may be due to abnormal activity of the MAPkinase or, conversely, may be due to decreased activity of a corresponding phosphatase. To investigate this, we incubated LA-N-5 neuroblastoma cells, which are known to contain τ protein, with the specific phosphatase inhibitor okadaic acid [1]. At low concentrations, okadaic acid is a specific inhibitor of phosphatase 2A, a Ser/Thre phosphatase found in all mammalian cells [11]. At higher concentrations, okadaic acid also inhibits phosphatase type 1 [11]. We found that LA-N-5 cells, after incubation with okadaic acid, express the AT8 epitope, while the complementary BT2 epitope, which is detectable in untreated cells, weakened. Our results suggest that phosphatases are implicated in the regulation of the phosphorylation state of τ.

Human neuroblastoma LA-N-5 cells (courtesy of Dr. Seeger, Children's Hospital Los Angeles, UCLA) were grown in 50% Dulbecco's modification of Eagle's medium (DMEM) /50% F-12 medium (Gibco, Ghent, Belgium) supplemented with 15% fetal bovine serum, 5 mM L-glutamine and 100 U/ml of penicillin/streptomycin [10]. The cells were cultured in a humidified incubator at 37°C, with 5% CO_2 in T-25 Falcon tissue culture flasks (Becton-Dickinson, Erembodegem, Belgium).

Okadaic acid was added to the medium to obtain final concentrations of 0.5 μM up to 2 μM. The cells were exposed to okadaic acid for time periods of 30 min up to 5 days. Some cell cultures were transiently exposed to

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okadaic acid to test for reversibility of the effect on τ phosphorylation. For this, parallel cell cultures were used, one of which was untreated, a second was exposed to okadaic acid for 2 h and then extracted for gel electrophoresis and a third culture was exposed for 2 h, then washed with medium and left to recover for 20 h. At the conclusion of each experiment the cells were processed as described [1]. Polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli with minor modifications [7]. The antibodies used for this study included: AT8, a monoclonal antibody directed to phosphorylated Ser 199/202 of the human τ protein and BT2, directed to the same epitope in its unphosphorylated condition. The epitope recognized by BT2 is similar to that of tau-1 and we have found that both compete for binding to normal human τ (not shown). Blotted proteins were incubated with primary antibody overnight at 4°C and detected with the avidin biotinylated peroxidase technique (Biogenex, San Ramon, CA). For immunocytochemical evaluation, cell suspensions were washed twice in Tris-buffered saline (TBS), smeared on poly-L-lysine coated glass slides, air dried and fixed in cold methanol (−20°C) for 2 min. After fixation, the cells were immunostained with the avidin-biotin complex technique. Controls consisted of omission of primary antibody, substituting the primary antibody by a non-specific antibody and dephosphorylation. For this, slides were pretreated for 10 min with 400 μg/ml trypsin (Gibco) in 0.05 M Tris buffer with 0.03 M NaCl and 0.02 M $CaCl_2$ at pH 7.6 and 37°C. They were then incubated with 400 μg/ml of bovine intestinal mucosa alkaline phosphatase type VII-L (Sigma) for 24 h at 33°C.

Extracts of LA-N-5 neuroblastoma cells, treated with okadaic acid for 30 minutes up to 8 h, were immunoblotted. LA-N-5 cells treated with okadaic acid for 2 h showed strong immunoreactivity with AT8, suggesting that in these cells, Ser 199 and/or 202 were phosphorylated (Fig. 1). The AT8 immunoreactivity was evident in cells treated with okadaic acid for as short as 45 min, but the intensity of immunoreactivity increased to reach a peak at 2 h and was stable after this (not shown). Also, immunoreactivity with AT8 was more intense on the extracts of cells that had been treated with 1 μM of okadaic acid, than in the cells that had been treated with the lower concentration of 0.5 μM. Untreated cell extracts showed a set of 4 isoforms of τ strongly immunoreactivity with BT2 (Fig. 1, lane 5), indicating the absence of phosphorylation of the Ser 199/202 epitope. Treated cells, on the other hand, showed decreased presence of the BT2 immunoreactivity (Fig. 1, lane 6). This decrease was evident in cells that had been exposed for only 30 min, but further decreased in cells

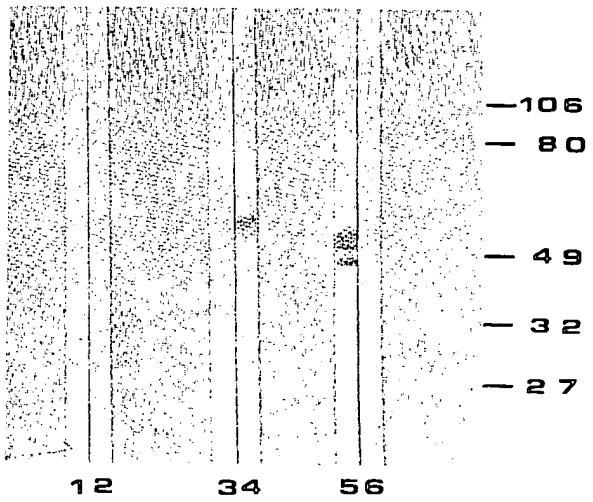

Fig. 1. Lane 1: immunoblot of untreated LA-N-5 neuroblastoma cells, non-specific control antibody. Lane 2: cells exposed to okadaic acid, control antibody. Lane 3: untreated cells, monoclonal antibody AT8 directed to phosphorylated Ser 199/202 of human τ. Lane 4: neuroblastoma cells after 2 h exposure to okadaic acid show a distinct band in the 60 kDa range, indicating the presence of the AT8 epitope. Lane 5: untreated cells show several isoforms of τ labelled with the BT2 monoclonal antibody directed to Ser 199/202 epitope in the non-phosphorylated state. Lane 6: there is a decrease in immunostaining of the BT2 immunoreactive bands after exposure to okadaic acid.

that had been exposed longer. As opposed to the cells extracted after 2 h of exposure, cells that had been transiently exposed to okadaic acid and then left to recover, showed no AT8 immunoreactivity (not shown).

Untreated cells showed strong immunoreactivity with BT2 and remained unlabelled by AT8 (Fig. 2a,c). After incubation with okadaic acid, some of the neuroblastoma cells detached from the substrate and neurites were retracted. Also, neuroblastoma cells exposed to okadaic acid were smaller than the untreated cells, but viability was not affected. After treatment, BT2 immunoreactivity was much weakened, while AT8 became positive (Fig. 2b,d). This AT8 immunoreactivity was completely abolished by prior incubation with alkaline phosphatase, while BT2 immunoreactivity was not affected.

The aberrant phosphorylation of the microtubule associated protein τ in AD is characterized by the presence of several epitopes unique to PHF-τ : the AT8 epitope is formed only when Ser 199/202 are phosphorylated. Complementary antibodies, such as BT2 or tau-1 recognize this epitope only in its normal, non-phosphorylated condition.

Studies on the abnormal phosphorylation of τ in Alzheimer's disease have hitherto always focused on kinases as the primary agent in producing these epitopes. However, the phosphorylation state of a protein is always the result of a dynamic balance between phosphorylation and dephosphorylation. Recently, it was demonstrated

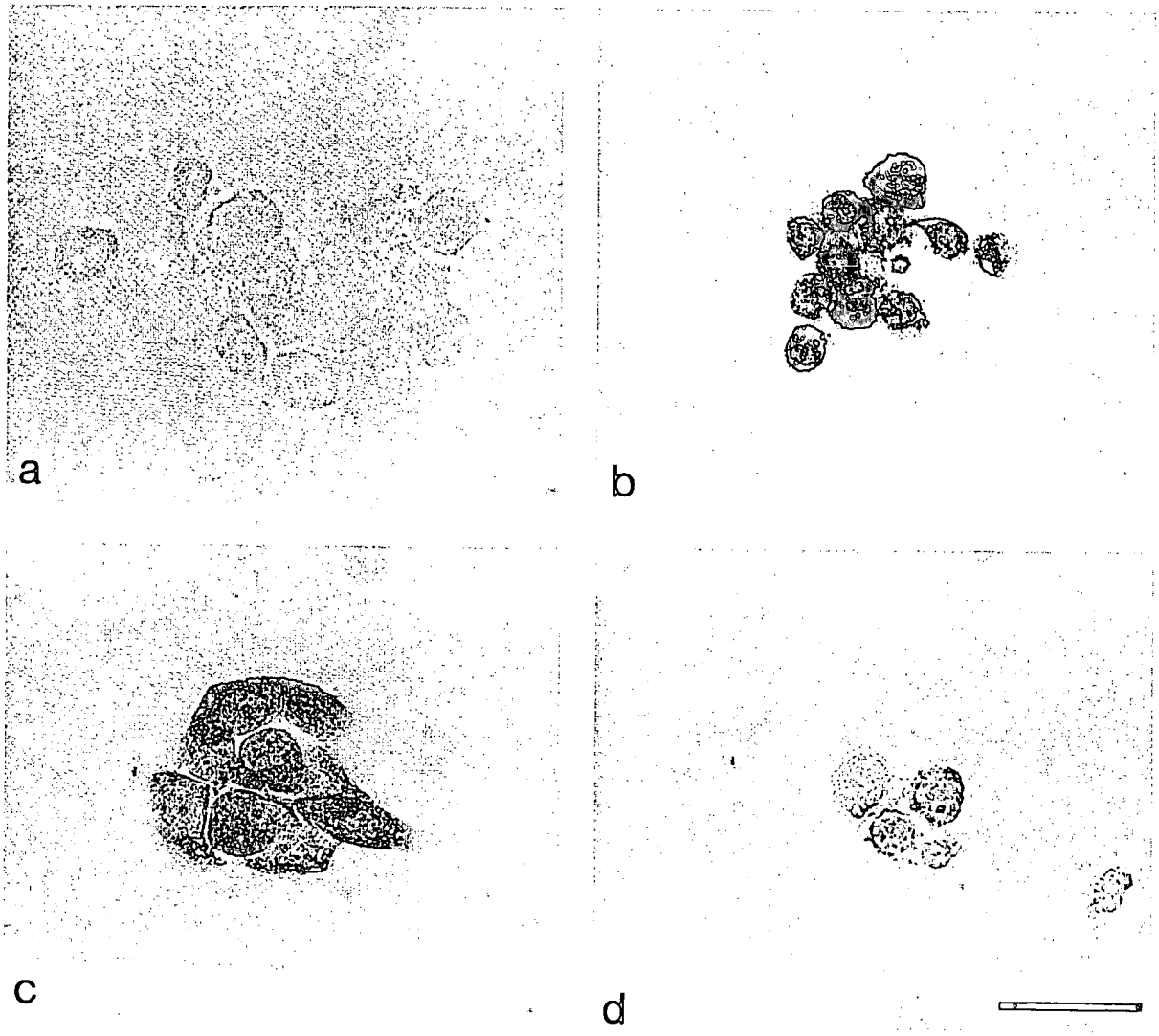

Fig. 2. a: untreated LA-N-5 neuroblastoma cells show no immunoreactivity with the monoclonal antibody AT8. b: after exposure to okadaic acid, there is distinct cytoplasmic AT8 immunoreactivity. c: untreated cells show intense BT2 immunoreactivity. d: the BT2 immunostaining is less intense after exposure to okadaic acid. Streptavidin biotinylated peroxidase technique. Magnification identical in a–d; bar = 20 μm.

that a MAPkinase, also known as ERK2, could generate the AT8 epitope [4]. The activity of this MAPkinase is regulated by tyrosine phosphorylation and a number of extracellular signals that enhance MAPkinase activity have recently been reviewed [8]. Our results suggest that inhibition of phosphatase activity may also play an important role in the emergence of phosphorylated PHF-τ epitopes. Okadaic acid is a specific inhibitor of phosphatase 2A, a Ser/Thre phosphatase which acts on the α-subunit of phosphorylase kinase a, phosphorylase a and also on the microtubule associated protein τ [11]. However, at higher concentrations (2 μM and higher), okadaic acid also inhibits PP1 and PP2B.

Several cell culture models for the in vitro generation of PHF-like structures have been proposed [6]. The use of complementary antibodies BT2 and AT8 recognizing the normal and abnormally phosphorylated form of the Ser199/202 epitope, allows the study of progressive alterations of the microtubule associated protein τ. More importantly, incubation of LA-N-5 neuroblastoma cells with okadaic acid now provides a cellular model in which the generation of a well defined PHF-τ epitope can be investigated. Also, this cell model allows the screening of potential neuroprotective drugs.

1 Argasinski, A., Sternberg, H., Fingado, B. and Huynh, P., Doxorubicin affects Tau protein metabolism in human neuroblastoma cells, Neurochem. Res., 14 (1989) 927–931.

2 Biernat, J., Mandelkow, E.M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M. and Mandelkow, E., The switch of tau-protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region, EMBO J., 11 (1992) 1593–1597.

3 Binder, L.I., Frankfurther, A. and Rebhun, L.I., The distribution of

tau in the mammalian central nervous system, J. Cell Biol., 101 (1985) 1371–1378.

4 Drewes, G., Lichtenberg-Kraag, B., Döring, F., Mandelkow, E.-M., Biernat, J., Goris, J., Dorée, M. and Mandelkow, E., Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state, EMBO J., 11 (1992) 2131–2138.

5 Lee, V.M., Balin, B.J., Otvos, L.J. and Trojanowski, J.Q., A68: a major subunit of paired helical filaments and derivatized forms of normal Tau, Science, 251 (1991) 675–8.

6 Mattson, M.P., Antigenic changes similar to those seen in neurofibrillary tangles are elicited by glutamate and Ca^{2+} influx in cultured hippocampal neurons, Neuron, 4 (1990) 105–17.

7 Mercken, M., Vandermeeren, M., Lübke, U., Six, J., Boons, J.,Van de Voorde, A., Martin, J.-J. and Gheuens, J., Monoclonal antibodies with selective specificity for Alzheimer tau are directed against phosphatase sensitive epitopes, Acta Neuropathol., 84 (1992) 265–272.

8 Pelech, S.L. and Sanghera, J.S., MAP kinases: charting the regulatory pathways, Science, 257 (1992) 1355–1356.

9 Saitoh, T., Masliah, E., Jin, L.W., Cole, G.M., Wieloch, T. and Shapiro, I.P., Protein kinases and phosphorylation in neurologic disorders and cell death, Lab. Invest., 64 (1991) 596–616.

10 Seeger, R.C., Rosenblatt, H.M., Imai, K. and Ferrone, S., Common antigenic determinants on human melanoma, glioma, neuroblastoma and sarcoma cells defined with monoclonal antibodies, Cancer Res., 41 (1981) 2714–2717.

11 Shenolikar, S. and Nairn, A.C., Protein phosphatases — recent progress, Adv. Sec. Messenger Phosphoprotein Res., 23 (1991) 1–121.

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Neurobiol of Aging 1996 March-April, 17(2):235-41

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Article title	Abnormally phosphorylated tau in SY5Y human neuroblastoma cells
Article identifier	001457939510250M
Authors	Tanaka_T Iqbal_K Trenkner_E Liu_D_J Grundke_Iqbal_I
Journal title	FEBS Letters
ISSN	0014-5793
Publisher	Elsevier Netherlands
Year of publication	1995
Volume	360
Issue	1
Supplement	0
Page range	5-9
Number of pages	5
User name	Adonis
Cost centre	Development
PCC	$20.00
Date and time	Thursday, September 28, 2000 3:50:36 PM

FEBS 15133 FEBS Letters 360 (1995) 5–9

Abnormally phosphorylated tau in SY5Y human neuroblastoma cells

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Received 30 December 1994

Abstract **In Alzheimer disease (AD) the microtubule associated protein (MAP) tau is hyperphosphorylated at several sites. In the present study, like AD tau, tau in the human neuroblastoma SH-SY5Y was found to be hyperphosphorylated, at Ser-199/202, Thr-231, Ser-396 and Ser-404. However, in contrast to AD, the tau in SY5Y cells was not hyperphosphorylated at Ser-235 and there was only one tau isoform. Quantitative analysis revealed that approximately 80% of the SY5Y- tau was phosphorylated at Ser-199/202. The phosphorylated tau was deposited in perikarya and processes of the cells whereas most of the unphosphorylated (at Ser-199/202) tau was localized in the nucleus. Tau from the cell lysates did not bind to taxol-stabilized microtubules. In contrast, MAP1b and MAP2 from cell lysates bound to stabilized microtubules in vitro and were associated to the microtubule network in situ. Phosphorylation of tau at high levels, its inactivity with microtubules and its accumulation in SY5Y cells provide for the first time a cell model of cytoskeletal changes seen in AD.**

Key words: Alzheimer disease; Microtubule associated protein; Microtubule; Neuroblastoma cell; Protein phosphorylation; Tau; Tubulin

1. Introduction

A major biochemical abnormality in the Alzheimer disease (AD) brain is the abnormally hyperphosphorylated microtubule associated protein (MAP) tau [1–3]. The abnormal tau is the major protein subunit of the paired helical filament (PHF) [1,3–5] and it is in this form that tau accumulates as neurofibrillary tangles in the perikaryon, in the neuropil threads [6] of the neuropil and in the dystrophic neurites of neuritic (senile) plaques. In addition, the abnormal tau also accumulates in normal appearing neurons in amorphous aggregates intermixed with a few short PHF, forming the so-called 'stage 0' tangles [7,8]. Unlike the mature neurofibrillary tangles, the stage 0 tangles are only poorly argentophilic and are not stained by thioflavin-S or Congo red. Recently, hyperphosphorylated tau (AD P-tau) was isolated from AD brain that, although not ubiquitinated and not polymerized into PHF, sediments at 27,000 to 200,000 × *g*, is soluble in buffers after denaturation with urea and has phosphate levels comparable to those of the PHF [9]. Furthermore, like PHF, the AD P-tau is phosphorylated at several sites and does not promote in vitro microtubule assembly unless dephosphorylated with alkaline phosphatase [10,11].

Previously it was not known whether the altered solubility characteristics of AD-P tau and its negative effect on microtubule assembly observed in vitro also occur in the living cell. In the present study we show that approximately 80% of tau in the human neuroblastoma cell line SH-SY5Y is hyperphosphorylated at the Tau-1 epitope, and, like the AD P-tau is hyperphosphorylated at least at three other sites. Furthermore, the abnormal tau, like in AD, accumulates in the cell body and does not seem to be biologically active since it is neither associated to the cellular microtubule network in situ nor in vitro binds to taxol-stabilized microtubules. However, unlike the abnormal tau in AD, the SY5Y-tau is not phosphorylated at Ser-235. The microtubule network itself seems to be intact in the SY5Y cells and is supported by MAP2 and MAP1b.

2. Materials and methods

2.1. Cells and proteins

SH-SY5Y human neuroblastoma cells were obtained from Dr. June L. Biedler (Sloan Kettering Institute, NY). Cells were cultured up to about 70% confluence in 35-mm diameter dishes employing D-MEM/F-12 medium (Gibco BRL, Gaithersburg, MD) (5% CO_2; 37°C) supplemented with 5% fetal calf serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The cells were fed every three days. In some cases the cells were deprived of nutrient by keeping them in the same medium without feeding for two to four additional days. For Western blots and immunoassays, the cells were collected from each dish by pipetting up and down, were lysed in 100 μl of 2% SDS/μ-mercaptoethanol, heated in a boiling water bath for 5 min and sonicated in a water bath for 10 min. The protein concentrations were assayed by the modified Lowry method of Bensadoun and Weinstein [12]. In some cases tau was immunoprecipitated from the cell lysates with polyclonal anti-tau antibody, 92e [13] bound to agarose linked protein G (Pierce, Rockford, IL).

Tau protein from normal human brain and soluble abnormally phosphorylated tau from Alzheimer disease (AD P-tau) brain were prepared as previously described [9]. Sources and dilutions of anti-tau antibodies employed are shown in Table 1. Other antibodies employed were DM1A (anti-α tubulin, 1:1000, Sigma, St. Lois, MO), YL1/2 (anti-tyrosinated tubulin, 1:500, Sera Lab, Westbury, NY), anti-MAP1b (1:400, Amersham, Arlington Heights, IL), and anti-MAP2 [14] (1:1000; a gift from Dr. R.B. Vallee, Shrewsbury, MA). Immunoblots were performed as previously described [1].

To determine the isoforms of tau protein in SY5Y cells, the cell lysates were first dialysed against 100 mM Tris, pH 8.0, 1 mM EGTA, 0.1 mM EDTA, 1 mM $MgCl_2$, and protease inhibitors (5 μg/ml aprotinin, 5 μg/ml Leupoptin, 2 μg/ml pepstatin and 1 mM PMSF) and then treated with 100 U/ml alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IL) at 37°C for 8 h. Recombinant tau 23 and 24 [15] expressed in *E. coli* were employed as M_r markers.

2.2. Radioimmuno-dot-blot assay

Triplicate samples of cell lysates containing 1 μg and 3 μg of total protein in 5 μl were applied to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and dried at 37°C for 30 min. The tau contents were determined using Tau-1 and ^{125}I-labeled anti-mouse IgG (Amersham, Arlington Heights, IL) with or without prior dephosphorylation of the protein on the membrane with alkaline phosphatase as previously described [16]. Purified recombinant tau 39 was employed for the standard curve in the immunoassay.

2.3. Indirect immunofluorescence microscopy

SH-SY5Y cells were cultured in Lab-Tec slides (Nunc, Naperville,

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IL) and, fixed in 4% formaldehyde in 100 mM HEPES, pH 6.8, 0.2% Triton X-100. 5 mM EGTA and 10 mM $MgCl_2$ [17]. The cells were post-fixed with −20°C methanol for 5 min, washed with phosphate buffered saline, blocked in 2% BSA/ Tris buffered saline and incubated with primary antibodies followed by FITC-labeled secondary antibodies (Cappel, Durham, NC). The slides were mounted in Vector-Shield mounting media (Vector Lab. Inc., Burlingame, CA). Fixation of the cells in −20°C methanol instead of formalin resulted in immunostaining of comparable intensity.

2.4. Taxol stabilized microtubules

Twice cycled microtubules from rat brain [18] were cold-disassembled and employed to prepare MAP-free tubulin by phosphocellulose chromatography [19]. Taxol stabilized microtubules were then prepared by incubating 1 mg tubulin/ml with 20 μM Taxol at 37°C for 30 min.

2.5. Microtubule binding assay

The cells (~1 × 10^7) were lysed in 100 mM PIPES, pH 6.8, 0.1% Triton-X 100, 2 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium vanadate and protease inhibitors (see above), and centrifuged at 200,000 × *g* for 30 min. The supernatant (900 μg protein) was incubated with 100 μg of taxol-stabilized microtubules and 10 μM taxol at 37°C for 30 min and centrifuged at 70,000 × *g* for 45 min. The supernatant containing the unbound proteins and the pellet of stabilized microtubules and microtubule bound proteins were analyzed by immunoblots.

3. Results

3.1. Tau in SY5Y cells is hyperphosphorylated at several sites

Western blots developed with anti-tau antibodies demonstrated the expression of tau protein in SH-SY5Y human neuroblastoma cells (Fig. 1). AT8 (P Ser-199/202), PHF-1 (P Ser-396), SMI-31 (P Ser-396 and P Ser-404) and M4 (P Thr-231) antibodies all of which react with phosphorylated epitopes of tau, but not with normal tau, reacted with tau in SY5Y cell lysate. Tau-1 antibody that only recognizes its epitope if it is not phosphorylated (Ser-199/Ser-202), stained only one faint band on the Western blot but strongly reacted with a series of polypeptides in the 51–63 kDa range after pretreatment of the blots with alkaline phosphatase. In contrast, antibody SMI33 (Ser-235) stained the tau polypeptides, and the pretreatment of the blots with alkaline phosphatase had no apparent effect on the intensity, indicating that SY5Y-tau is not phosphorylated at Ser-235 (data not shown). SY5Y-tau dephosphorylated in vitro was stained as a single band and co-migrated with the recombinant tau 23, the human tau isoform with three repeats of tubulin binding domains and no N-terminal inserts (same as fetal tau) [15].

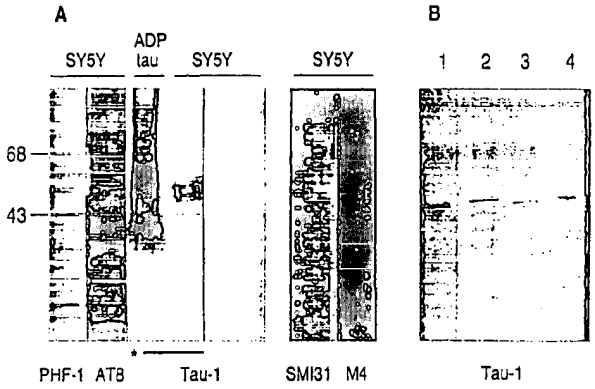

Fig. 1. Phosphorylation sites and M_r of SH-SY5Y tau. (A) 100 μg of cell lysates were applied to each lane; AD P-tau was used at 2 μg/lane. *Indicates alkaline phosphatase treatment of the Western blot prior to the application of the antibodies. In the case of SMI31 and M4, samples immunoprecipitated with tau antibody 92e were applied to avoid non-specific staining. Phosphorylation of SY5Y-tau at Ser-199/202 is indicated by the reaction with AT8 as well as by a marked increase in tau staining with Tau-1 after dephosphorylation of the blot. The reaction of PHF-1 and SMI 31 with SY5Y tau indicates phosphorylation at Ser-396/404. The reaction of M4 with SY5Y-tau indicates phosphorylation of tau at Thr-231. Not shown in this figure, SY5Y tau is not significantly phosphorylated at Ser-235 since there was no increase of tau staining with antibodies SMI33 after dephosphorylation of the blots. (B) 50 μg of cell lysates dephosphorylated in vitro were applied to lane 1 and 0.2 μg/each of recombinant tau 23 and 24 were applied to lanes 2 and 3 respectively. Apparent M_r weight of tau 23 and 24 are 48 kDa and 52 kDa, respectively [15]. Dephosphorylated SY5Y-tau (lane 1) was visualized as a single band that corresponded to tau 23, the shortest human tau isoform. SY5Y-tau in the untreated cell lysate (100 μg) in lane 4 has slower mobility than dephosphorylated SY5Y tau in lane 1.

3.2. Most of the tau is phosphorylated at Ser-199/202

To determine what percentage of tau was hyperphosphorylated in the SY5Y cells, levels of tau in cell lysate were determined by radioimmuno-dot-blot assay using Tau-1 with and without pretreatment of the blots with alkaline phosphatase. A 5–6-fold increase in the tau levels after dephosphorylation was observed (Fig. 2), while the levels of tau isolated from control brains were not affected by the phosphatase treatment (data not shown). From three independent experiments the percentage of tau abnormally phosphorylated at the

Table 1
Source and dilution of the tau antibody

Ab	Type	Epitope	Phosphorylated (P)/Not-Phosphorylated (N)	Dilution	References
Tau antibodies					
Tau-1	mono	Ser 199/202	N	1:50,000	34
AT8	mono	Ser 199/202	P	1:50	35
M4	mono	Thr 231	P	1:2,000	36
SMI33	mono	Ser 235	N	1:500	37
SMI31	mono	Ser 269/404	P	1:100	37
PHF1	mono	Ser 396	P	1:100	38
92e	poly	–	–	1:5*	13

*Dilution used for immunoprecipitation.

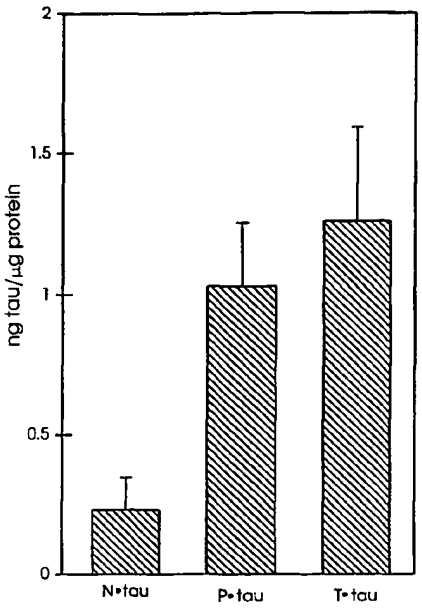

Fig. 2. Levels of tau hyperphosphorylated at Ser-199/202. Levels of tau in SY5Y lysates were estimated by comparison with defined amounts of recombinant tau 39. Blots treated (T · tau = total tau) or untreated with alkaline phosphatase (N · tau = tau not phosphorylated at the Tau-1 epitope) were exposed to Tau-1 antibody [^{125}I]anti-mouse IgG. The value for P · tau (phosphorylated at the Tau-1 epitope) was determined by subtraction of the value obtained for N · tau from that of T · tau. The data were obtained from three independent experiments.

Tau-1 epitope was determined to be 82% ± 10 of the total tau level of 1.3 ± 0.2 ng/µg lysate protein.

3.3. *Phosphorylated tau accumulates in the cytoplasm in SY5Y cells*

The association of tau to the microtubule network in SY5Y neuroblastoma cells was examined by indirect immunofluorescence (Figs. 3, 4). A dense microtubule network was seen with anti-tubulin antibody in cell bodies and neurites, (Fig. 3d) whereas no fibrillar staining by Tau-1, AT8 or PHF-1 antibodies (Fig. 3a–c) was observed. Tau-1 labeling was seen as several bright dots in the nucleus while in the cytosol only a faint background staining was observed (Fig. 3a). In contrast, strong immunoreactivity of AT8 (P Ser-199/202) was found in the cytoplasm and in the several neuronal processes (Fig. 3b). No binding to fibrous structures was apparent. PHF-1 (P Ser-396) stained brightly fluorescent deposits along the cytoplasmic seam around the unstained nucleus (Fig. 3c). Mitotic cells were most intensely labeled with PHF-1. In contrast to tau, anti-MAP1b and anti-MAP2 antibodies stained the intracellular fibrous network, and in addition several neuronal processes of the SY5Y cells (Fig. 3e).

When cells were cultured at suboptimal conditions, i.e. without changing the medium for 5 days to 7 days instead of 3 days, large accumulations of phosphorylated tau were observed in the cytoplasm, that were strongly immunoreactive with PHF-1 and AT8 (Fig. 4a,b). Apparently, the microtubule network of these cells was intact since it was labeled both with mAb DMIA to α tubulin and with YL1/2 antibody to the labile microtubule population (Fig. 4c,d).

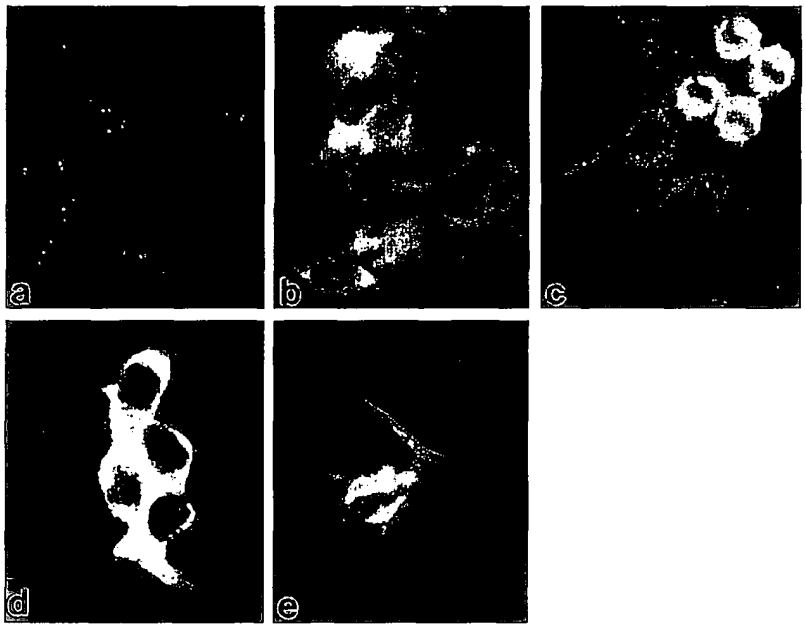

Fig. 3. Immunofluorescent staining of formalin fixed SH-SY5Y cells with antibodies to (a–c) tau: (a) Tau-1, (b) AT-8, and (c) PHF-1, (d) tubulin and (e) MAP1b. The microtubule network was stained with antibodies to tubulin, MAP1b and, not shown here, MAP2. No microtubule staining was observed with any of the tau antibodies.

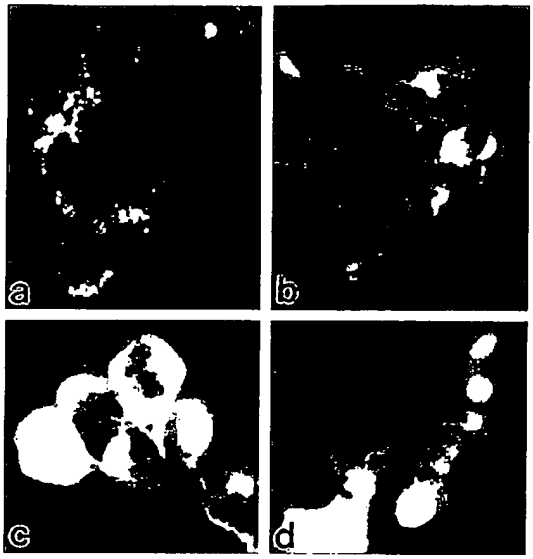

Fig. 4. Immunofluorescent staining of SH-SY5Y cells that were for 5 days in culture without medium change. Antibodies to hyperphosphorylated tau: AT-8 (a), PHF-1 (b), and to tubulin: DM1A (c), YL1/2 (d) were employed. The microtubule network appeared slightly less dense than in Fig. 3 while tau aggregation appeared more prominent. Not shown in this figure, a similar pattern was observed after 7 days of nutrient deprivation.

3.4. *Most SY5Y tau does not bind to microtubules*

To determine whether tau was biologically active in binding to microtubules, the 200,000 × *g* extract of the SY5Y cells was incubated with taxol-polymerized microtubules, and the microtubule bound and unbound proteins were separated into pellet and supernatant, respectively. The immunoblots of these fractions revealed that almost all of MAP1b and MAP2 bound but most of the SY5Y-tau did not bind to the microtubules (Fig. 5).

4. Discussion

In Alzheimer disease 75–85% of the MAP tau, including all six isoforms, is altered both in its degree of phosphorylation and its solubility-characteristics [1,3,9,13,16]. In vitro studies have shown that the abnormal hyperphosphorylation of tau renders it biologically inactive [10,11,20]. In the present study we show for the first time that tau in SY5Y cells is phosphorylated at five of six of the abnormal phosphorylation sites examined, and also does not bind to microtubules. These findings suggest that like in AD, tau in SY5Y cells is hyperphosphorylated, has minimal biological activity and accumulates in the cell cytoplasm. Hyperphosphorylation of tau similar to that in AD has also been observed in fetal rat and human brains [21,22]. However, immunocytochemical studies indicate that in contrast to AD, tau in the fetal brain is immobilized only in a relatively small number of neurons [23].

Of the total SY5Y-tau, about 80% is hyperphosphorylated at the Tau-1 epitope. Similarly high phosphorylation levels of the Tau-1 epitope have been previously demonstrated both in the soluble abnormally phosphorylated tau and the PHF-tau in AD [1,9]. Whether the degree of phosphorylation at Thr-231, Ser-396 and Ser-404 is as high as at the Tau-1 epitope, cannot be determined with the antibodies available to date. Furthermore, it is not as yet known at what additional sites, if any, tau in SY5Y cells is phosphorylated. However, it appears that the hyperphosphorylation of SY5Y tau is sufficient to make it not bind to microtubules and accumulate in the living cell.

Generally, endogenous tau in cells as well as tau in cells transfected with tau cDNA have been shown to decorate a fibrous network, presumably microtubules [24–26]. In contrast, in the present study most of the hyperphosphorylated tau is deposited in the perikarya of SY5Y cells and is not bound to the microtubule network. These findings suggest that the hyperphosphorylation of tau might be the cause for both its accumulation and biological inactivity. The greater deposition of tau in SY5Y cells in nutrient deprived medium might be due to a decrease in pH. In vitro self-assembly of tau or its fragments has been reported at pH 4.5–5.5 [27–29].

Although most of the cytoplasmic tau was phosphorylated at the Tau-1 epitope, tau in the nucleus was not hyperphosphorylated. Tau has been shown previously to colocalize with the nucleolus of several neuroblastoma cells [30]. There might be unknown regulatory mechanism that affect the localization of tau by phosphorylation. For example, certain proteins such as the nuclear factor of activated T-cells (NF-AT) and MAP kinase are known to be translocated to the nucleus depending upon their phosphorylation state [31,32].

The lack of microtubules in neurons with neurofibrillary tangles [33] as well as the in vitro inhibition of tau-mediated microtubule assembly by Alzheimer tau [11] suggests a potential role of the hyperphosphorylated tau in the microtubule defect in AD. In SY5Y cells, on the other hand, the microtubule system is apparently functional despite the presence of mostly hyperphosphorylated tau. A reason for this discrepancy might be that MAP1b, which is most probably expressed at higher levels in the readily dividing SY5Y cells than in the non-divid-

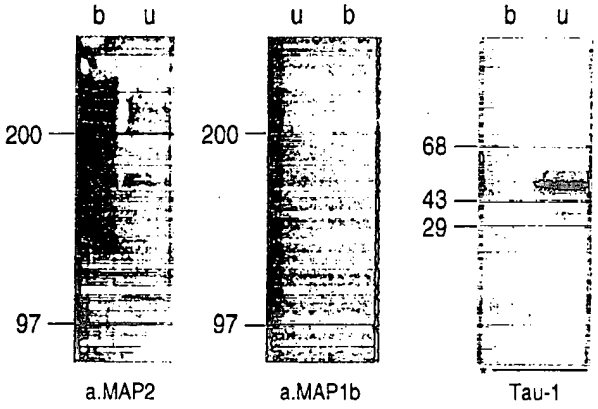

Fig. 5. Microtubule binding of MAPs from SY5Y cell extract. The 200,000 × *g* extract of the SY5Y cells was incubated with taxol-stabilized microtubules and the MAPs bound or unbound to microtubules were examined by Western blots as described in section 2. For blots 10% (20 μg) of the pellet containing the microtubules and the bound proteins and 10% (100 μg) of the supernatant representing the unbound proteins were applied per lane, and the MAPs that were bound (b) or unbound (u) to the microtubules were studied with the appropriate antibodies; *Indicates alkaline phosphatase treatment of the immunoblot before antibody application. Although MAP1b and MAP2 bound to the microtubules, most of tau did not bind.

ing neurons of the brain, together with MAP2 compensates for the adverse effect of the hyperphosphorylated tau. Alternatively, SY5Y tau is probably not as hyperphosphorylated as AD tau to cause the breakdown of the microtubule system. The differences in the degree of tau phosphorylation between SY5Y cells and AD brain, are already indicated from its lack of phosphorylation at Ser-235 in the cultured cells. Nevertheless the present study shows the inactivity of hyperphosphorylated tau with microtubules and its accumulation in cultured cells i.e. outside of the human brain.

Acknowledgements: We thank Dr. L.I. Binder, University of Alabama, Birmingham, AL; Dr. S. Greenberg, W.M. Burke Medical Research Institute, White Plains, NY; Innogenetics, Zwijndrecht, Belgium; Dr. Y. Ihara, Tokyo University, Japan; Dr. R.B. Vallee, Worcester Foundation for Experimental Biology, Shrewsbury, MA; and Dr. M. Goedert, MRC Laboratory of Molecular Biology, Cambridge, UK for generously supplying antibodies Tau-1, PHF-1, AT8, M4 and anti-MAP2 and human tau cDNA, respectively. We also thank Dr. A. Alonso, Dr. N. Haque, Ms. T. Zaidi, and Ms. Y.-C. Tung, for providing purified tubulin, recombinant human tau, AD P-tau and normal human tau; Ms. J. Lopez and P. Reginald for typing the manuscript. This work was supported in part by funds from the New York State Office of Mental Retardation and Developmental Disabilities, National institute of Health Grant NS 18105, AG05892, AG08076 and Zenith Award (K.I.) from the Alzheimer's Association, USA.

References

[1] Grundke-Iqbal, I., Iqbal, K., Tung, Y.-C., Quinlan, M., Wisniewski, H.M. and Binder, L.I. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.
[2] Iqbal, K., Grundke-Iqbal, I., Zaidi, T., Merz, P.A., Wen, G.Y., Shaikh, S., Wisniewski, H.M., Alafuzoff, I. and Winblad, B. (1986) Lancet 2, 421–426
[3] Iqbal, K., Grundke-Iqbal, I., Smith, A.J., George, L., Tung, Y.-C. and Zaidi, T. (1989) Proc. Natl. Acad. Sci. USA 86, 5646–5650.
[4] Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.-C., Zaidi, M.S. and Wisniewski, H.M. (1986) J. Biol. Chem. 261, 6084–6089.
[5] Lee, V.M.-Y., Balin, B.J., Otvos Jr., L. and Trojanowski, J.Q. (1991) Science 251, 675–678.
[6] Braak, H., Braak, E., Grundke-Iqbal, I. and Iqbal, K. (1986) Neurosci. Lett. 65, 351–355.
[7] Bancher, C., Brunner, C., Lassmann, H., Budka, H., Jellinger, K., Wiche, G., Seitelberger, F., Grundke-Iqbal, I., Iqbal, K. and Wisniewski, H.M. (1989) Brain Res. 477, 90–99.
[8] Bancher, C., Grundke-Iqbal, I., Iqbal, K., Fried, V.A., Smith, H.T. and Wisniewski, H.M. (1991) Brain Res. 539, 11–18.
[9] Köpke, E., Tung, Y.-C., Shaikh, S., Alonso, A., Iqbal, K. and Grundke-Iqbal, I. (1993) J. Biol. Chem. 268, 24374–24384.
[10] Iqbal, K., Zaidi, T., Bancher, C. and Grundke-Iqbal, I. (1994) FEBS Lett. 349, 104–108,
[11] Alonso, A., Zaidi, T., Grundke-Iqbal, I. and Iqbal, K. (1994) Proc. Natl. Acad. Sci. USA 91, 5562–5566.
[12] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[13] Grundke-Iqbal, I., Vorbrodt, A.W., Iqbal, K., Tung, Y.-C., Wang, G.-P. and Wisniewski, H.M. (1988) Mol. Brain Res. 4, 43–52.
[14] Bloom, G.S. and Vallee, R.B. (1983) J. Cell. Biol. 96, 1523–1531.
[15] Goedert, M. and Jakes R. (1990) EMBO J. 9, 4225–4230.
[16] Khatoon, S., Grundke-Iqbal, I. and Iqbal, K. (1992) J. Neurochem. 59, 750–753.
[17] Takemura, R., Okabe, S., Umeyama, T., Kanai, Y., Cowan, N. and Hirokawa, N. (1992) J. Cell Sci. 103, 953–964.
[18] Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) Proc. Natl. Acad. Sci. USA 70, 765–768.
[19] Sloboda, R.D. and Rosenbaum, J.L. (1979) Biochemistry 18, 48–55.
[20] Lindwall, G., and Cole, R.D. (1984) J. Biol. Chem. 259, 5301–5305.
[21] Brion, J.-P., Smith, C., Couck, A.-M., Gallo, J.-M. and Anderton, B.H. (1993) J. Neurochem. 61, 2071–80.
[22] Watanabe, A., Hasegawa, M., Suzuki, M., Takio, K., Morishima-Kawashima, M., Titani, K., Arai, T., Kosik, K.S. and Ihara, Y. (1993) J. Biol. Chem. 268, 25712–25717.
[23] Wolozin, B., Scicufella, A. and Davies, P. (1988) Proc. Natl. Acad. Sci. USA 85, 6202–6206.
[24] Kanai, Y., Takemura, R., Oshima, T., Mori, H., Ihara, Y., Yanagisawa, M., Masaki, T. and Hirokawa, N. (1989) J. Cell. Biol. 109, 1173–1184.
[25] Barlow, S., Gonzalez-Garay, M.L., West, R.R., Olmsted, J.B. and Cabral, F. (1994) J. Cell. Biol. 126, 1017–1029.
[26] Drubin, D., Kobayashi, S. and Kirshner, S. in: Dynamic Aspects of Microtubule Biology (Soifer, D. eds.) Ann. NY Acad. Sci., vol. 466, pp. 257–268, NY Acad. Sci., New York.
[27] Ruben, G.C., Iqbal, K., Grundke-Iqbal, I., Wisniewski, H.M., Ciardelli, T.L. and Johnson Jr., J.E. (1991) J. Biol. Chem. 266, 22019–22027.
[28] Crowther, R.A., Olsen, O.F., Jakes, R. and Goedert, M. (1992) FEBS Lett. 309, 199–202.
[29] Wille, H., Drewes, G., Biernat, J., Mandelkow, E.-M. and Mandelkow, E. (1992) J. Cell. Biol. 118, 573–584.
[30] Loomis, P.A., Howard, T.H., Castleberry, R.P. and Binder, L.I. (1990) Proc. Natl. Acad. Sci. USA 87, 8422–8426.
[31] Liu, J. (1993) Immunol. Today 14, 290–295.
[32] Lenormand, P., Sardet, C., Pagès, G., L'Allmain, G., Brunet, A. and Pouysségur (1993) J. Cell. Biol. 122, 1079–1088.
[33] Dustin, P. and Flament-Durant, I. (1982) in: Axoplasmic Transport and Pathology (Weiss and Gorio eds.) pp. 131–136, Springer-Verlag, New York.
[34] Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985) J. Cell. Biol. 101, 1371–1378.
[35] Biernat, J., Mandelkow, E.-M., Schröter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M. and Mandelkow, M. (1992) EMBO J. 11, 1593–1597.
[36] Hasegawa, M., Watanabe, A., Takio, K., Suzuki, M., Arai, T., Titani, K. and Ihara, Y. (1993) J. Neurochem. 60, 2068–2077.
[37] Lichtenberg-Kraag, B., Mandelkow, E.-M., Biernat, J., Steiner, B., Schröter, C., Gustke, N., Meyer, H.E. and Mandelkow, E. (1992). Proc. Natl. Acad. Sci. USA 89, 5384–5388.
[38] Greenberg, S.G., Davies, P., Schein, J.D. and Binder, L.I. (1992) J. Biol. Chem. 267, 564–569.

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Neurobiol of Aging 1996 March-April, 17(2):235-41

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Article title	Dephosphorylation of microtubule-associated protein tau by protein phosphatase-1 and -2C and its implication in Alzheimer disease
Article identifier	001457939410311H
Authors	Gong_C_X Grundke_Iqbal_I Damuni_Z Iqbal_K
Journal title	FEBS Letters
ISSN	0014-5793
Publisher	Elsevier Netherlands
Year of publication	1994
Volume	341
Issue	1
Supplement	0
Page range	94-98
Number of pages	5
User name	Adonis
Cost centre	Development
PCC	$20.00
Date and time	Thursday, September 28, 2000 3:51:33 PM

ELSEVIER

FEBS Letters 341 (1994) 94–98

FEBS 13756

FEBS LETTERS

Dephosphorylation of microtubule-associated protein tau by protein phosphatase-1 and -2C and its implication in Alzheimer disease

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Received 24 January 1994

Abstract

Microtubule-associated protein tau is abnormally hyperphosphorylated and forms the major protein subunit of paired helical filaments (PHF) in Alzheimer disease brains. The abnormally phosphorylated sites Ser-199, Ser-202, Ser-396 and Ser-404 but not Ser-46 and Ser-235 of Alzheimer tau were found to be dephosphorylated by protein phosphatase-1 and this dephosphorylation was activated by Mn^{2+}. In contrast, protein phosphatase-2C did not dephosphorylate any of these sites. Both protein phosphatase-1 and -2C had high activities towards [^{32}P]tau phosphorylated by cAMP-dependent protein kinase. These results suggest that both protein phosphatase-1 and -2C might be associated with normal phosphorylation state of tau, but only the former and not the latter phosphatase is involved in its abnormal phosphorylation in Alzheimer disease.

Key words: Tau; Protein phosphatase; Dephosphorylation; Alzheimer disease

1. Introduction

Microtubule-associated protein tau is a family of polypeptides of apparent molecular weight 50,000 to 64,000 Da that are the products of alternate splicing of a single gene [1,2]. Tau promotes the assembly of tubulin into microtubules and stabilizes their structure. Tau is a phosphoprotein and phosphorylation regulates its microtubule assembly promoting activity [3]. Recently, more research interest has been focused on tau phosphorylation because abnormally hyperphosphorylated tau has been proven to be the main protein component of paired helical filaments (PHF) which is one of the most characteristic cellular and molecular changes in Alzheimer disease (AD) brain [4]. PHF-tau and in vitro phosphorylated tau have lower activity to stimulate microtubule assembly as compared to normal tau, while dephosphorylation of PHF-tau by alkaline phosphatase can recover this activity [5,6].

Tau normally contains 2–3 mol of phosphate per mol of the protein, whereas it contains 5–9 mol of phosphate per mole of the protein in AD brain [7–9]. So far nine abnormal phosphorylation sites of PHF-tau have been identified which are not phosphorylated in normal adult brain. They are Ser-46 [10], Thr-123 [11], Ser-199 [4,12,13], Ser-202 [4,12,13], Thr-231 [14], Ser-235 [14,15], Ser-262 [14], Ser-396 [15,16], and Ser-404 [17] as numbered according to the largest isoform of human tau, tau_{441} [2]. It seems there is a disfunction of tau phosphorylation/dephosphorylation system which leads to the abnormal hyperphosphorylation of tau in AD brain. Therefore, it is of great interest to identify the protein kinases and protein phosphatases which are involved in the reversible process of tau phosphorylation.

Phosphoseryl and phosphothreonyl protein phosphatases (PP) have been classified by Cohen and Ingebritsen into four main types, i.e. PP-1, PP-2A, PP-2B and PP-2C [18]. These protein phosphatases are present in significant concentrations in human brain [19]. PP-2A and PP-2B have been shown to dephosphorylate tau phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase and cAMP-dependent protein kinase (PKA) [20,21]. Recently, we found that PP-2A (Gong et al., manuscript submitted for publication) and PP-2B [22] also dephosphorylate Alzheimer tau at the abnormal phosphorylation sites. In this study, we describe the activities of the other two types of protein phosphatases, PP-1 and PP- 2C, towards tau phosphorylated by PKA and Alzheimer disease abnormally phosphorylated tau (AD P-tau).

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Abbreviations: AD, Alzheimer disease; AD P-tau, Alzheimer disease abnormally phosphorylated tau; PHF, paired helical filaments; PKA, cAMP-dependent protein kinase; PP, protein phosphatase.

SSDI 0014-5793(94)00141-H

2. Materials and methods

2.1. Materials

Phosphorylase kinase was purified from rabbit skeletal muscle by the method of Cohen [23]. PKA was purchased from Sigma, St. Louis, MO, USA. Rabbit skeletal muscle PP-1 was purchased from Upstate Biotechnology Inc., Lake Placid, NY. PP-2B (holoenzyme) was purified from bovine brain according to the method of Sharma et al. [24]. PP-2C was purified from bovine kidney as described previously [25]. Polyclonal antibodies 102c were raised as previously reported [10]. Monoclonal antibodies Tau-1 and PHF-1 were kindly provided by Drs. L.I. Binder [26] and S. Greenberg [27], respectively; SMI33, SMI31, goat anti-mouse IgG and peroxidase-anti-peroxidase complex were purchased from Sternberger Monoclonals Inc., Baltimore, MD. Alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Bio-Rad, Hercules, CA.

2.2. Isolation of tau

AD P-tau and normal human tau were isolated from autopsied brains of a 70-year-old male with Alzheimer disease and a 51-year-old male normal case, respectively [9]. Briefly, AD P-tau was isolated from a non-neurofibrillary tangle pool, the 27,000 × *g* to 200,000 × *g* fraction of the Alzheimer brain homogenate by extraction in 8 M urea, followed by dialysis against Tris buffer. This AD P-tau enriched fraction is readily soluble in buffer and abnormally phosphorylated as PHF-tau [9]. Normal human tau was purified from the 35–45% ammonium sulfate precipitates of 200,000 × *g* brain supernatant, followed by acid treatment (pH 2.7) and chromatography on a phosphocellulose column (Cellulose Phosphate P11, Whatman).

2.3. Preparation of [^{32}P]phosphorylase kinase and determination of protein phosphatase activities

[^{32}P]Phosphorylase kinase (1.9 mol ^{32}P incorporated/335,000 × *g*) phosphorylated by PKA was prepared as reported previously [19]. The activities of PP-1, PP-2B and PP-2C were measured by counting the radioactivity released from [^{32}P]substrate as previously described [19]. The reaction mixtures contained 50 mM Tris, pH 7.0, 20 mM β-mercaptoethanol, 1.0 mM $MnCl_2$ and 1.0 μM [^{32}P]phosphorylase kinase for PP-1. For PP-2B and PP-2C activities, $MnCl_2$ was substituted by 1.0 mM $CaCl_2$ and 1.0 μM calmodulin, and 10 mM $MgCl_2$, respectively. One unit of protein phosphatase activity is defined as that amount which catalyzes the release of 1.0 nmol phosphate per min from [^{32}P]phosphorylase kinase at 30°C.

2.4. Treatment of AD P-tau with protein phosphatases

Unless otherwise stated, dephosphorylation of AD P-tau was carried out at 30°C in 50 mM Tris, pH 7.0, 10 mM β-mercaptoethanol, 0.1 mg/ml BSA, 50 μg/ml AD P-tau and PP-1, PP-2B or PP-2C. In some experiments, several effectors were added in the reaction mixture (see section 3). The reaction was started by addition of the enzyme. After appropriate incubation times (see figure legends), reactions were stopped by addition of 5 volumes of cold acetone to precipitate proteins. The precipitated protein samples were dissolved in SDS-PAGE sample buffer and heated at 95°C for 4 min, followed by 10% SDS-PAGE. Immunoblotting was carried out as described previously [4]. The primary antibodies for immunoblotting and their epitopes have all been previously characterized. They are phosphorylation-dependent as well as site-specific. Briefly, antibodies 102c [10], Tau-1 [4,12,13] and SMI33 [15] recognize dephosphorylated form of tau at sites Ser-46, Ser-199/Ser-202 and Ser-235, respectively. Antibodies SMI31 [15] and PHF-1 [28] recognize tau phosphorylated at Ser-396/Ser-404 and Ser-396, respectively. These antibodies were used at dilutions of 0.4 μg/ml for 102c, 1:100 for SMI31, 1:500 for SMI33 and PHF-1, and 1:500,000 for Tau-1.

2.5. Preparation of [^{32}P]tau and dephosphorylation of [^{32}P]tau by protein phosphatases

Tau purified from normal human brain was phosphorylated with [^{32}P]ATP by PKA as described by Scott et al. [29]. About 2 mol ^{32}P/mol tau was incorporated by PKA. Dephosphorylation of [^{32}P]tau by PP-1, PP-2B and PP-2C was carried out employing the same conditions as those described above for the dephosphorylation of AD P-tau. The phosphatase activities were measured by counting the radioactivity released from [^{32}P]tau as previously described [19].

3. Results

3.1. Definition of PP-1 and PP-2C activities and their modulation by Mn^{2+} and Mg^{2+}

To observe and compare the potential dephosphorylation of AD P-tau by various protein phosphatases, the same amount of enzyme activity of each phosphatase should be used. Hence we employed [^{32}P]phosphorylase kinase as a substrate to standardize the activities of PP-1 and PP-2C. PP-1 and PP-2C activities are modulated by cations and each enzyme preparation responds differently to divalent cations [30]. We therefore determined PP-1 and PP-2C activities in the absence and presence of either Mn^{2+} or Mg^{2+} using [^{32}P]phosphorylase kinase as a substrate. As shown in Fig. 1, PP-1 was activated by 1.0 mM Mn^{2+} but inhibited by 10 mM Mg^{2+}. PP-2C was Mg^{2+}-or Mn^{2+}-dependent, and no activity was detected in the absence of Mg^{2+} or Mn^{2+}. Highest activities were obtained using 1.0 mM Mn^{2+} for PP-1 and 10 mM Mg^{2+} for PP-2C. Hence these conditions were used to study the in vitro dephosphorylation of AD P-tau and PKA-phosphorylated tau.

3.2. Treatment of AD P-tau with PP-1 and PP-2C

We have previously found that PP-2A dephosphorylated abnormal phosphorylation sites Ser-46, Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau (Gong et al., manuscript submitted for publication), and

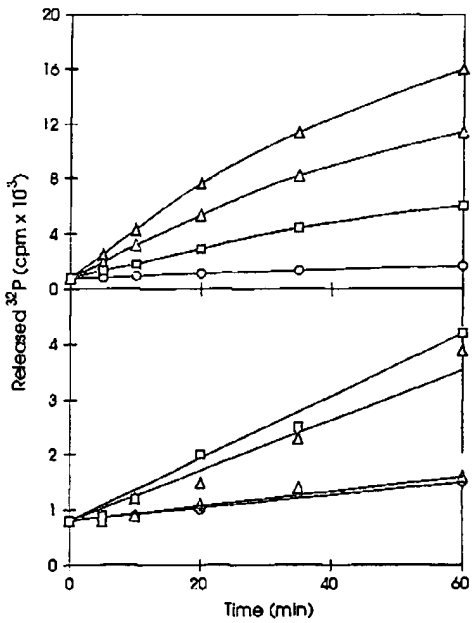

Fig. 1. PP-1 (upper panel) and PP-2C (lower panel) activities in the presence or absence of various divalent metal ions. Dephosphorylation reactions were carried out using [^{32}P]phosphorylase kinase as substrate as described in section 2, and in the presence of 1.0 mM EDTA (△), 1.0 mM $MnCl_2$ (▲) or 10 mM $MgCl_2$ (□). The open circles (○) indicate assays in the absence of the protein phosphatases.

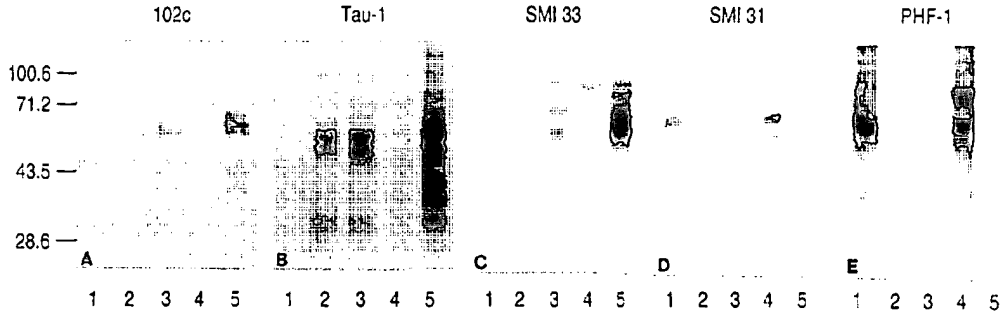

Fig. 2. Dephosphorylation of AD P-tau by PP-1, PP-2B and PP-2C. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 2.0 units/ml PP-1 (lane 2), PP-2B (lane 3) or PP-2C (lane 4) at 30°C for 60 min as described in Materials and Methods; lane 5 shows untreated normal human tau for comparison. Reaction mixtures for PP-1 and PP-2C also contained 1.0 mM $MnCl_2$ and 10 mM $MgCl_2$, respectively. For PP-2B, 1.0 μM calmodulin, 1.0 mM $CaCl_2$ and 1.0 mM $MnCl_2$ were included. Five phosphorylation-dependent antibodies were used for immunoblotting as shown above each panel to monitor dephosphorylation of the specific sites of AD P-tau. 102c (A), Tau-1 (B) and SMI33 (C) recognize dephosphorylated forms, whereas SMI31 (D) and PHF-1 (E) recognize phosphorylated forms of tau at specific sites as described in section 2. Molecular weight (kDa) markers are indicated at left margin of the figure. SMI33, a monoclonal antibody to neurofilament heavy/medium subunits, crossreacts with tau unphosphorylated at Ser-235 [15]. In lane 4, panel C, the 88 kDa protein band stained prominently without a corresponding staining in the tau region is probably not tau.

that in addition to above sites, PP-2B also dephosphorylated another abnormal phosphorylation site, Ser-235 [22]. In the present study, using immunoblots with five site-specific phosphorylation-dependent antibodies which recognize six abnormal phosphorylation sites of AD P-tau, we have further examined whether PP-1 and PP-2C can also dephosphorylate these abnormal phosphorylation sites at optimum in vitro conditions. PP-2B was employed as a positive control. We found that PP-1 unmasked the epitope of antibody Tau-1 and blocked the epitopes of antibodies SMI31 and PHF-1, but failed to unblock the epitopes of antibodies 102c and SMI33 (Fig. 2). PP-2C did not change any of these epitopes. These results indicate that PP-1 dephosphorylates abnormal phosphorylation sites Ser-199, Ser-202, Ser-396 and Ser-404 but not Ser-46 and Ser-235 of AD P-tau. Whereas PP-2C had no effect on dephosphorylation of above sites.

The rate of dephosphorylation of Ser-199/Ser-202, Ser-396/Ser-404 and Ser-396 of AD P-tau by PP-1 was determined using immunoblots with Tau-1, SMI31 and PHF-1, respectively. The time course showed a rapid change of epitopes of AD P-tau towards these three antibodies (Fig. 3). Within 20–30 min incubation of AD P-tau with PP-1, staining of Tau-1 became maximal and those of both PHF-1 and SMI31 disappeared completely. These results suggested that the four phosphorylation sites of AD P-tau can be easily hydrolyzed by PP-1.

We have also investigated the dephosphorylation of AD P-tau at various conditions (Fig. 4). In the absence of metal (1.0 mM EDTA present), PP-1 could also dephosphorylate AD P-tau at Ser-396 but the activity was low. Dephosphorylation of AD P-tau by PP-1 was strongly activated by 1.0 mM Mn^{2+} but inhibited by 10 mM Mg^{2+}. We further investigated the required concentration of Mn^{2+} for this activation. The activation was observed at 10 μM Mn^{2+} and it reached maximum at about 100 μM Mn^{2+} (Fig. 5); the physiological level of Mn^{2+} is 5–11 μM in brain [33].

3.3. Dephosphorylation of [^{32}P]tau by PP-1 and PP-2C

Tau protein is known to be phosphorylated in vitro at Ser-214, Ser-324, Ser-356, Ser-409 and Ser-416 by PKA [29]. So far none of these sites have been reported to be abnormally phosphorylated in Alzheimer disease brain, but they may be involved in normal phosphorylation of tau. We therefore asked whether these non-abnormal

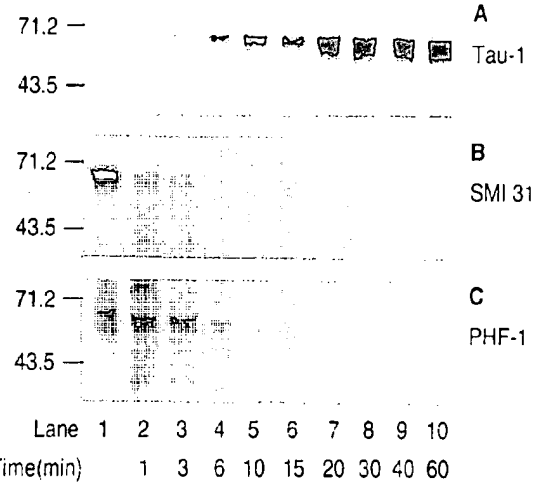

Fig. 3. Time course of dephosphorylation of AD P-tau by PP-1. Immunoblots of AD P-tau were carried out after incubation either without (lane 1) or with 1.0 unit/ml PP-1 as described in Fig. 2 at 30°C for different time intervals (lanes 2–10). Phosphorylation-dependent antibodies Tau-1 (A), SMI31 (B) and PHF-1 (C) were used to monitor the dephosphorylation. Molecular weight (kDa) markers are indicated at the left of each panel.

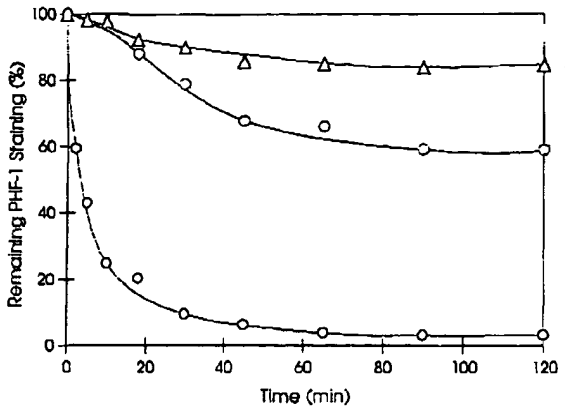

Fig. 4. Effect of Mn^{2+} and Mg^{2+} on dephosphorylation of AD P-tau by PP-1. AD P-tau was incubated with 1.0 unit/ml PP-1 in the presence of either 1.0 mM EDTA (○), 1.0 mM Mn^{2+} (○) or 10 mM Mg^{2+} (△) at 30°C for different time intervals as described in section 2. After incubation, AD P-tau was subjected to immunoblotting with monoclonal antibody PHF-1 which stains only phosphorylated forms of tau, followed by densitometric scanning. Dephosphorylation is expressed by percentage of remaining PHF-1 staining.

phosphorylation sites of tau can be dephosphorylated by either PP-1 and PP-2C. Interestingly, even though PP-1, PP-2B and PP-2C had obviously different effects on dephosphorylation of abnormal phosphorylation sites of AD P-tau, they had similar high activities towards [^{32}P]tau phosphorylated by PKA (Fig. 6).

4. Discussion

In order to examine whether there is a defect of protein phosphatase(s) that might be involved in the abnormal phosphorylation of tau in AD brain, it is essential to identify which protein phosphatase(s) can dephosphorylate the AD P-tau. We have recently found that PP-2A and PP-2B rapidly dephosphorylate AD P-tau in vitro ([22] and Gong et al. manuscript submitted for publication). The present study shows that PP-1 also dephosphorylates AD P-tau at some of the sites whereas PP-2C has no activity towards any of the sites studied. Although three of four types of protein phosphatases can dephosphorylate AD P-tau, they have different site specificities. Six of nine known abnormal phosphorylation sites of AD P-tau have been examined in these studies. They are Ser-46, Ser-199, Ser-202, Ser-235, Ser-396 and Ser-404. All of them can be dephosphorylated by PP-2B; PP-2A can dephosphorylate all except S-235; and PP-1 dephosphorylates Ser-199, Ser-202, Ser-396 and Ser-404 but neither Ser-46 nor Ser-235. Hence at least four abnormal phosphorylation sites, Ser-199, Ser-202, Ser-396 and Ser-404, can be dephosphorylated by the three enzymes, PP-1, PP-2A and PP-2B. These results indicate that the regulation of phosphorylation level of tau is very complex and more than one protein phosphatase might be involved in hyperphosphorylation of tau in AD.

When [^{32}P]phosphorylase kinase was used as a substrate to determine protein phosphatase activities, PP-1 activity was about 20-fold higher than PP-2C activity in human brain extracts [19]. In the present study, also using [^{32}P]phosphorylase kinase as a substrate to define the activities of both PP-1 and PP-2C, 1.0 unit/ml of PP-1 almost completely dephosphorylated Ser-199, Ser-202, Ser-396 and Ser-404 of AD P-tau in 20–30 min, but 2.0 units/ml of PP-2C did not dephosphorylate AD P-tau at any sites studied at the optimal in vitro conditions. Taken together, these results suggest that AD P-tau is not a substrate for PP-2C.

Tau isolated from adult brain normally contains 2–3 mol of phosphate per mol of the protein [7–9]. However, neither the phosphorylation sites nor the responsive kinase(s) have yet been fully elucidated. Normal tau might be partially phosphorylated at Ser-202 and Ser-404 [31,32], but to date other sites have not been excluded to be phosphorylated. PKA is known to phosphorylate tau at Ser-214, Ser-234, Ser-356, Ser-409 and Ser-416 [29]. PP-2C as well as PP-1 and PP-2B can release about 80% radioactivity from PKA-phosphorylated [^{32}P]tau in 60 min, suggesting that these phosphatases dephosphorylate most of these phosphorylation sites of tau. Therefore, even if it is not involved in abnormal phosphorylation of Alzheimer tau, PP-2C may be associated with the regulation of phosphorylation level of normal tau.

The present study also shows that PKA-phosphorylated tau and AD P-tau serve as different substrates for protein phosphatases. AD P-tau can be dephosphorylated by PP-1, PP-2A and PP-2B but not by

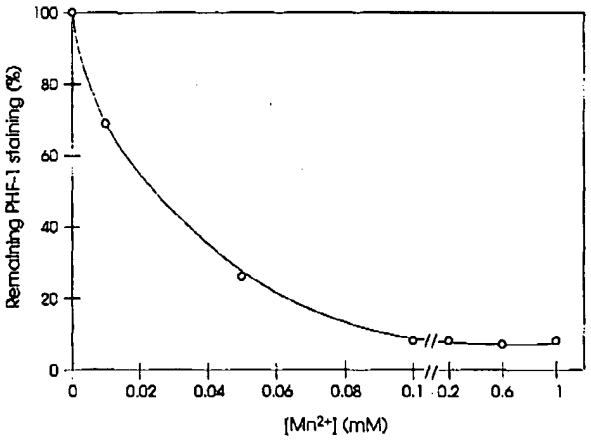

Fig. 5. Dephosphorylation of AD P-tau by PP-1 at various concentrations of Mn^{2+}. AD P-tau was incubated with 1.0 unit/ml PP-1 in the presence of various concentrations of $MnCl_2$ at 30°C for 60 min as described in section 2. After incubation, AD P-tau was subjected to immunoblotting with PHF-1 (which stains only tau phosphorylated at Ser-396) followed by densitometric scanning. Dephosphorylation is expressed by percentage of remaining PHF-1 staining.

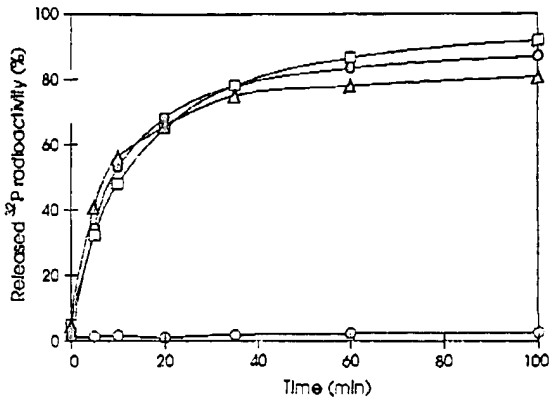

Fig. 6. Dephosphorylation of PKA-phosphorylated tau by PP-1, PP-2B and PP-2C. PKA-phosphorylated tau (0.1 mg/ml) was incubated either without (○) or with 0.4 unit/ml of PP-1 (●), PP-2B (□) or PP-2C (△) at 30°C for different time intervals as described in section 2. The reaction mixtures also included 1.0 mM $MnCl_2$ for PP-1, 1.0 mM $CaCl_2$, 1.0 μM calmodulin and 1.0 mM $MnCl_2$ for PP-2B, and 10 mM $MgCl_2$ for PP-2C.

PP-2C, whereas PKA-phosphorylated tau is almost an equally good substrate for PP-1, PP-2B and PP-2C. Whether the completely different behavior of AD P-tau and PKA-phosphorylated tau as a substrate for PP-2C is due to different phosphorylation sites, and/or due to different protein conformations remains to be investigated.

Acknowledgements: We would like to thank Dr. L.I. Binder, University of Alabama, Birmingham, AL, for providing Tau-1 antibody, Dr. S.G. Greenberg, Burke Medical Research Institute, White Plains, NY, for providing PHF-1, Dr. T.J. Singh of our laboratory for a gift of PP-2B, and Y.-C. Tung and T. Zaidi of our laboratory for isolation of tau. Autopsied brain specimens were generously supplied by the Netherlands Brain Bank, Amsterdam, the Netherlands; and the Brain Tissue Resource Center, Belmont, MA, USA. This work was supported by the New York State Institute for Basic Research in Developmental Disabilities, grants NS18105, AG05892, AG08076, and AG04220 from the National Institutes of Health, Zenith Award (to K. Iqbal) from Alzheimer's Association, USA, and grant DMB- 9019882 from the National Science Foundation (to Z. Damuni).

References

[1] Himmler, A., Drechsel, D., Kirschner, M.W. and Martin, D.W. Jr. (1989) Mol. Cell. Biol. 9, 1381–1388.
[2] Goedert, M., Spillantini, M.G., Rutherford, D. and Growther, R.A. (1989) Neuron 3, 519–526.
[3] Lindwall, G., and Cole, R.D. (1984) J. Biol. Chem. 259, 5301–5305.
[4] Grundke-Iqbal, I., Iqbal K., Tung, Y.-C., Quinlan, M., Wisnieswski, H. and Binder, L. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.
[5] Iqbal, K., Grundke-Iqbal, I., Zaidi, T., Merz, P.A., Wen, G.Y., Shaikh, S.S., Wisniewski, H.M., Alafuzoff, I. and Winblad, B. (1986) Lancet 2, 421–426.
[6] Iqbal, K., Koepke-Secundo, E. and Grundke-Iqbal, I. (1991) J. Neuropathol. Exp. Neurol. 50, 316 (Abstract).
[7] Selden, S.C. and Pollard, T.D. (1983) J. Biol. Chem. 258, 7064–7071.
[8] Ksiezak-Reding, H., Liu, W.-K. and Yen, S.-H. (1992) Brain Res. 597, 209–219.
[9] Köpke, E., Tung, Y.-C., Shaikh, S., Alonso, A.C., Iqbal, K. and Grundke-Iqbal, I. (1993) J. Biol. Chem. 268, 24374–24384.
[10] Iqbal, K., Grundke-Iqbal, I., Smith, A.J., George, L., Tung, Y.-C. and Zaidi, T. (1989) Proc. Natl. Acad. Sci. USA 86, 5646–5650.
[11] Brion, J.P., Hanger, D.P., Bruce, M.T., Couck, A.M., Flament-Durand, J. and Anderton, B.T. (1991) Biochem. J. 273, 127–133.
[12] Biernat, J., Mandelkow, E.-M., Schroter, C., Lichtenberg-Kraag, B., Steiner, B., Berling, B., Meyer, H., Mercken, M., Vandermeeren, A., Goedert, M. and Mandelkow, E. (1992) EMBO J. 11, 1593–1597.
[13] Szendrei, G.I., Lee, V.M.-Y. and Otvos, L.Jr. (1993) J. Neurosci. Res. 34, 243–249.
[14] Hasegawa, M., Morishima-Kawashima, M., Takio, K., Suzuki, M., Titani, K. and Ihara, Y. (1992) J. Biol. Chem. 267, 17047–17054.
[15] Lichtenberg-Kraag, B., Mandelkow, E.-M., Biernat, J., Steiner, B., Schröter, C., Gustke, N., Meyer, H.E. and Mandelkow, E. (1992) Proc. Natl. Acad. Sci. USA 89, 5384–5388.
[16] Lee, V.M.Y., Balin, B.J., Otvos, L. and Trojanowski, J.Q. (1991) Science 251, 675–678.
[17] Kanemaru, K., Takio, K., Miura, R. Titani, K. and Ihara, Y. (1992) J. Neurochem. 58, 1667–1675.
[18] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[19] Gong, C.-X., Singh, T.J., Grundke-Iqbal, I. and Iqbal, K. (1993) J. Neurochem. 61, 921–927.
[20] Yamamoto, H., Saitoh, Y., Fukunaga, K., Nishimurura, H. and Miyamoto, E. (1988) J. Neurochem. 50, 1614–1623.
[21] Goto, S., Yamamoto, H., Fukunaga, K., Iwasa, T., Matsukado, Y. and Miyamoto, E. (1985) J. Neurochem. 45, 276–283.
[22] Gong, C.-X., Singh, T.J., Grundke-Iqbal, I. and Iqbal, K. (1994) J. Neurochem. 62, 803–806.
[23] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[24] Sharma, R.K., Taylor, W.A. and Wang, J.H. (1983) Methods Enzymol. 102, 210–219.
[25] Amick, G.D., Reddy, S.A.G. and Damuni, Z. (1992) Biochem. J. 287, 1019–1022.
[26] Binder, L.I., Frankfurter, A. and Rebhun, L.I. (1985) J. Biol. Chem. 101, 1371–1378.
[27] Greenberg, S.G., Davies, P., Schein, J.D. and Binder, L.I. (1992) J. Biol. Chem. 267, 564–569.
[28] Lang, E., Szendrei, G.I., Lee, V.M.-Y. and Otvos, L., Jr. (1992) Biochem. Biophys. Res. Commun. 187, 783–790.
[29] Scott, C.W., Spreen, R.C., Herman, J.L., Chow, F.P. and Davison, M.D. (1993) J. Biol. Chem. 268, 1166–1173.
[30] Ballou, L.M. and Fischer, E.H. (1987) The Enzymes 17, 311–361.
[31] Arioka, M., Tsukamoto, M., Ishiguro, K., Kato, R., Sato, K., Imahori, K. and Uchida, T. (1993) J. Neurochem. 60, 461–468.
[32] Poulter, L., Barratt, D., Scott, C.W. and Caputo, C.B. (1993) J. Biol. Chem. 268, 9636–9644.
[33] Friberg, L., Nordberg, G.F. and Vouk, V.B., Eds. (1986) Handbook on the Toxicology of Methods. Vol. 2, pp. 264–366, Elsevier, Amsterdam, New York.

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Neurobiology of Aging, Vol. 17, No. 2, pp. 235–241, 1996

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Neurodegenerative Changes Including Altered Tau Phosphorylation and Neurofilament Immunoreactivity in Mice Transgenic for the Serine/Threonine Kinase Mos

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Received 17 April 1995; Revised 3 July 1995; Accepted 17 September 1995

JAMES, N. D., D. R. DAVIES, J. SINDON, D. P. HANGER, J.-P. BRION, C. C. J. MILLER, M. P. ROSENBERG, B. H. ANDERTON AND F. PROPST. *Neurodegenerative changes including altered tau phosphorylation and neurofilament immunoreactivity in mice transgenic for the serine/threonine kinase Mos.* NEUROBIOL AGING 17(2) 235–241, 1996.—Transgenic mice expressing the oncogenic protein-serine/threonine kinase Mos at high levels in the brain display progressive neuronal degeneration and gliosis. Gliosis developed in parallel with the onset of postnatal transgene expression and led to a dramatic increase in the number of astrocytes positive for GFAP, vimentin, and possibly tau. Interestingly, vimentin is normally expressed only in immature or neoplastic astrocytes, but appears to be induced to high levels in *Mos*-transgenic, mature astrocytes. Mos can activate mitogen activated protein kinase (MAPK) and MAPK has been implicated in Alzheimer-type tau phosphorylation. In the *Mos*-transgenic brain we found increased levels of phosphorylation at one epitope on tau containing serines 199 and 202 (numbering according to human tau), a pattern similar but not identical to that found in Alzheimer's disease. In addition, *Mos*-transgenic mice express a novel neurofilament-related protein that might be a proteolytic neurofilament heavy chain degradation product. These results suggest that activation of protein phosphorylation in neurons can result in changes in cytoskeletal proteins that might contribute to neuronal degeneration.

Mos Protooncogene Progressive neuronal degeneration Gliosis Tau phosphorylation Transgenic mouse Neurofilament GFAP Vimentin

SEVERAL common neurodegenerative diseases have characteristic abnormalities of the cytoskeleton in vulnerable neurons. In Alzheimer's disease many neurons contain hyperphosphorylated tau in the form of paired helical filaments (PHF) (48). In senile dementia of the Lewy body type and in Parkinson's disease cortical neurons and neurons of the substantia nigra contain perikaryal spherical accumulations of neurofilaments (Lewy bodies) (37,52). A characteristic of motor neuron disease is the presence of axonal spheroids in motor neurons, and these, too, are neurofilamentous (29,49). It is frequently assumed that defective regulation of neurofilament phosphorylation is responsible for these various neurofilament pathologies, which is based upon the observation that the perikaryal neurofilament inclusion bodies are labeled with antibodies selective for phosphorylated neurofilament epitopes. Such epitopes are normally found only on axonal neurofilaments, perikaryal neurofilaments being predominantly dephosphorylated at those sites (32). We have, therefore, examined cytoskeletal proteins in the brains of transgenic mice overexpressing a protein kinase, namely, the protein-serine/threonine kinase encoded by the Mos protooncogene. These animals were of particular interest because they have been shown to exhibit neurodegenerative changes in response to Mos overexpression (40). Moreover, the Mos protein kinase has been shown to activate mitogen activated protein kinase (MAPK) (31,38,46). The latter enzyme is capable of phosphorylating in vitro cytoskeletal proteins such as the microtubule-associated protein tau at sites phosphorylated in PHF-tau found in Alzheimer's disease (15).

The *Mos* protooncogene encodes a protein-serine/threonine kinase (28,41,54) with a key function in the regulation of meiosis during maturation of vertebrate oocytes. It has been demonstrated

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that Mos is necessary and sufficient for the induction of maturation in *Xenopus* oocytes in vitro (16,42,53) and is responsible for the regulated progression of oocytes through meiotic divisions (43). Similar results have been reported for mouse oocytes (33,36,56) and have been confirmed in vivo in mice lacking *Mos* (11,18). In addition to its normal function, Mos has a potent oncogenic transforming activity in tissue culture cells (34). Both normal and transforming functions of Mos are believed to be mediated by the MAPK signal transduction pathway. It has been shown that Mos can activate MAPK, presumably through phosphorylation and activation of MAPK kinase (MAPKK) (31,38,46).

Transgenic mice carrying activated *Mos* transgenes display a range of neurological phenotypes including movement and posture abnormalities, tremors, progressive ataxia, and paralysis (39,40). Histopathological examination of the central nervous system (CNS) shows vacuolation of the neuropil, severe neuronal, and axonal degeneration, infiltration of the neuropil and meninges by inflammatory cells, and gliosis. In addition, abnormal neurons that stain positive for phosphorylated epitopes of neurofilaments in the perikaryon and abnormal astrocytes with large atypical nuclei are observed. These pathologic alterations correlate with overexpression of the *Mos* transgene in affected areas of the CNS as assessed by in situ hybridization. The lesions appear at around 1 month of age, increase in severity up to 5 months, but do not appear to be fatal, as affected mice will live to a normal life span.

Here we report that in the brains of *Mos*-transgenic mice we find increased levels of phosphorylation at one epitope on tau (equivalent to serine 202 of human tau). This pattern of phosphorylation is partially similar but not identical to that found on PHF-tau in Alzheimer's disease (2,24). We also found that *Mos*-transgenic mice but not nontransgenic mice express a protein species in the brain that is labeled by several antibodies to phosphorylated neurofilaments and might be a proteolytic degradation product of the neurofilament heavy chain (NF-H). Immunohistochemical studies of the transgenic brains with antibodies to amyloid β protein (Aβ) did not reveal the presence of amyloid deposits. These results suggest that activation of protein phosphorylation in neurons can result in changes in cytoskeletal proteins. Thus, transgenic mice expressing activated protein kinases in neurons may be a useful model for studying the participation of the cytoskeleton in the neurodegenerative process.

METHOD

Animals

c-*Mos*-transgenic mice of line 4 or line 3, both carrying the pTS74 transgene (40), and normal littermates or age- and sex-matched controls were used for this study. Where necessary, the genotype of mice was verified by Southern blot analysis (50) of tail DNA. All experiments described below were carried out using tissues from at least two individual transgenic animals at each state of postnatal development, and in each case the same results were obtained in all transgenic animals analyzed.

Antibodies

The rabbit polyclonal anti-GFAP serum I-2 was raised against purified mouse GFAP and characterized by comparison with a commercially available polyclonal anti-GFAP serum (Dako). For vimentin we used a monoclonal antivimentin antibody Vim 3B4 (Boehringer) and a rabbit polyclonal affinity-purified antibody (Euro-Diagnostic). Monoclonal antibody TAU1 (Boehringer) recognizes an epitope in tau provided that serines 199 and 202 (numbering according to human tau) are unphosphorylated (3) and the monoclonal antitau antibody AT8 (gift from Innogenetics, Ghent, Belgium) requires serine 202 to be phosphorylated to generate its epitope (2). B19 is a rabbit polyclonal antiserum raised against adult bovine tau proteins (5). SMI31, SMI33, and SMI34 (51) and 8D8, RT97, BF10, and RS18 are monoclonal antibodies directed against neurofilaments but crossreact with PHF-tau (7,10,25). For MAP2 analysis we used monoclonal antibodies HM-2 (Sigma) and AP18 (1). The anti-MAP2 antibody B9 was raised against rat high molecular weight MAP2 proteins (4). MAP1B was examined using monoclonal antibody G10 (17). The anti-β/A4 amyloid antibody is a rabbit polyclonal serum raised against a synthetic peptide corresponding to residues 12–28 of the β/A4 amyloid peptide.

Transgene mRNA Analysis

Total RNA was isolated from the brain as described (9) using the RNAzol reagent (Cinna/Biotecx) according to the manufacturer's protocol. RNA was analyzed using a ribonuclease protection assay supplied by Ambion according to the manufacturer's protocol. A radioactively labeled RNA probe was obtained by in vitro transcription of plasmid pGLMos (39), which was linearized with restriction endonuclease BspE1. Transcription of this template with Sp6 RNA polymerase in the presence of α-^{32}P-GTP yields a continuously labeled 540 nucleotide transcript of which 507 nucleotides are complementary to the 3' end of transgene mRNA.

Protein Preparation and Immunoblotting

Mice were sacrificed by cervical dislocation and the brain was dissected as rapidly as possible and placed on dry ice. The frozen brain was then weighed (wet weight) and either stored at −70°C or processed immediately. Samples were homogenized at 100 mg wet weight of tissue per ml in a buffer containing 150 mM Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.02% (w/v) bromophenol blue in a PT 10-35 Kinematica homogenizer (Brinkman, NY) followed by sonication and boiling. This procedure yields samples with a concentration of approximately 8 mg protein per ml. Equal volumes of all samples were fractionated on 8, 10, or 12% polyacrylamide gels (22) and transferred electrophoretically to supported nitrocellulose membranes (Hybond-C super, Amersham International, UK). Transferred blots were blocked with 5% (w/v) nonfat dry milk in TBST (10 mM Tris-HCl pH8, 150 mM NaCl, 0.05% (v/v) Tween 20) for either 2 h at room temperature or 4°C overnight. Following incubation with primary antibody for 1–2 h at room temperature or overnight at 4°C at the indicated dilutions in the same buffer detection was carried out by enhanced chemoluminescence (20) using the ECL immunoblotting detection system (Amersham International, UK) or by an alkaline phosphatase conjugate substrate kit (BioRad, UK) according to the manufacturer's protocols.

Immunohistochemistry

For immunohistochemistry, brains were removed and cut sagitally into two equivalent halfs. One-half was fixed in methacarn (methanol, chloroform, acetic acid) for 48 h, dehydrated, and embedded in paraffin. Tissue sections with a thickness of 7 μm were cut and processed for immunocytochemistry. The other half of the brain was fixed in 4% paraformaldehyde and sections were cut to 30 μm and processed similarly. The immunolabeling was performed using the peroxidase-antiperoxidase (PAP) method. Briefly, tissue sections were treated with H_2O_2 to inhibit endogenous peroxidase, incubated with the blocking solution (20% normal goat serum in TBS: 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4), and then overnight with the diluted primary antibody. Following incubation with goat antirabbit or antimouse antibody (Nordic) and a rabbit or mouse PAP complex (Nordic), peroxidase activity was

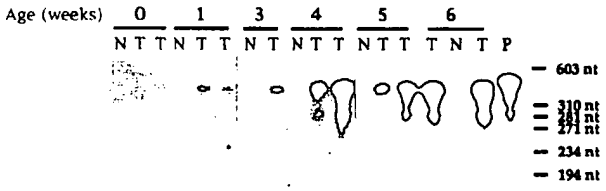


FIG. 1. Postnatal development of transgene expression in *Mos*-transgenic mice. Transgene mRNA expression was determined by an RNase protection assay of 10 μg of total brain RNA prepared from normal (N) or transgenic (T) littermates at the indicated ages. For each age the two transgenic samples with the highest and lowest levels of expression are shown (with the exception of 3-week-old animals where only one transgenic and one normal sample are shown). On the right, migration of size markers of the indicated length in nucleotides (nt) are shown. In samples of *Mos*-transgenic mice of at least 1 week of age the 540 nt riboprobe (P) is protected for a length of 507 nt, as expected. The intensity of the 507 nt band reflects the relative amount of transgene mRNA present in the sample. The low level of expression of the endogenous c-*Mos* gene reported for the brain (40) was not observed in this experiment due to the fact that total RNA instead of poly A+ was used.

revealed using diaminobenzidine as chromogen. Prior to immunolabelling a parallel set of sections was incubated for 20 h at 37°C with alkaline phosphatase from calf intestine (130 U/ml; Boehringer) diluted in 0.1 M Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonylfluoride, 10 mg/ml pepstatin, 10 mg/ml leupeptin. Prior to immunolabeling with the β/A4 amyloid antibody, sections were treated with formic acid for 10 min. As a control for the immunohistochemical detection of phosphorylated and unphosphorylated tau, hippocampal sections of patients with Alzheimer's disease were immunolabeled with the TAU1 and AT8 antibodies, with and without pretreatment with alkaline phosphatase. Dilution of primary antibodies in immunohistochemistry: B19, AT8, and anti-GFAP 1:1000; anti-MAP2, anti-β/A4, and SMI31, 1:500; TAU1, 1:250; antivimentin (Euro-Diagnostic), 1:50.

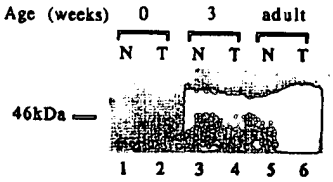
RESULTS

We have previously reported that mice transgenic for the c-*Mos* gene under the control of the strong Moloney murine sarcoma virus (MSV) promoter display severe, progressive degeneration of axons and neurons in the brain (40). This phenotype is observed in four independent lines of transgenics and is first apparent at 4 weeks of age. Subsequently, the severity of the lesions increases up to the age of 5 months. To correlate the appearance of these pathologic alterations with transgene expression during postnatal development we analyzed transgene mRNA levels during the first 6 weeks after birth. The results are shown in Fig. 1. Transgene expression is first detected at 1 week after birth and subsequently increases to reach high levels by 4 weeks of age when the brain pathology is first seen. We observed some variation of transgene mRNA levels between individual mice (for example, Fig. 1; 4- and 5-week-old transgenics), which might reflect slight differences in postnatal development.

The histopathological analysis of *Mos*-transgenic mice had revealed extensive astrocytosis detectable between 1 and 3 months of age (40). We, therefore, examined the expression of glial fibrillary acidic protein (GFAP) and vimentin, two marker proteins of glial intermediate filaments (13,14), during postnatal development. Whole brain protein extracts were prepared at different ages and analyzed by immunoblotting using antisera specific for GFAP or vimentin. In normal mice, GFAP expression was barely detectable at birth but increased to reach adult levels at around 3 weeks of age in agreement with published reports (13,14). In newborn transgenic animals, GFAP levels were as low as in normal mice, consistent with the absence of transgene expression at this age. However, by 3 weeks of age when transgene expression had started, GFAP levels were elevated in the transgenic brain, and in adult animals a dramatic increase in GFAP levels was observed (Fig. 2A). This increase was confirmed by immunohistochemical staining of the transgenic brain sections, which revealed a strong increase in the number of GFAP-positive cells (Fig. 4).

In normal newborn mice vimentin is expressed in glial cells but the levels rapidly decrease with increasing age and GFAP expression (13,14). In view of astrocytosis and concomitant increase in GFAP expression in *Mos*-transgenic mice we also examined vimentin expression during the postnatal period by immunoblotting. In normal mice, vimentin expression was highest in newborn animals, thereafter rapidly decreasing in the first 2 weeks of life and dropping to virtually undetectable levels by adulthood as has been described (13,14) (Fig. 2B). In the transgenic brain, in contrast, vimentin expression was initially identical to normal animals but rose steadily from 3 weeks of age to reach high levels in adult mice. In addition, in some but not all transgenic samples an additional band of apparent molecular weight 50 kDa was detectable (Fig. 2B). This protein was not analyzed further, but could be a degradation product of vimentin as has been reported for *Mos*-transformed tissue culture cells (47).

Given the progressive neuronal and axonal degeneration in the brain of *Mos*-transgenic mice and the observation that phosphorylation of cytoskeletal proteins might be involved in neuronal degeneration in general (2,24,32), we next examined the phosphorylation pattern of the microtubule-associated protein tau. The two antibodies chosen were TAU1 (3), which identifies an epitope in tau that requires serines 199 and 202 to be unphosphorylated, and



B: Vimentin

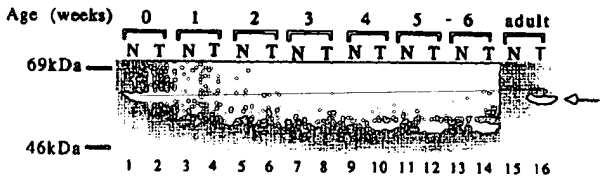


FIG. 2. Postnatal development of GFAP and vimentin expression in *Mos*-transgenic mice. Whole brain protein (50 μg protein/lane) prepared from normal (N) and transgenic (T) littermates at the indicated ages was analyzed by immunoblotting for the expression of GFAP using the I-2 anti-GFAP serum diluted 1:50 (panel A) and vimentin using the Vim 3B4 antivimentin serum diluted 1:200 (panel B). The 55 kDa full length vimentin band is indicated by the arrow. Immunoblot analysis using a commercially available anti-GFAP serum (Dako; diluted 1:200) yielded results identical to those shown in panel A. The samples correspond to the ones used for Fig. 1.

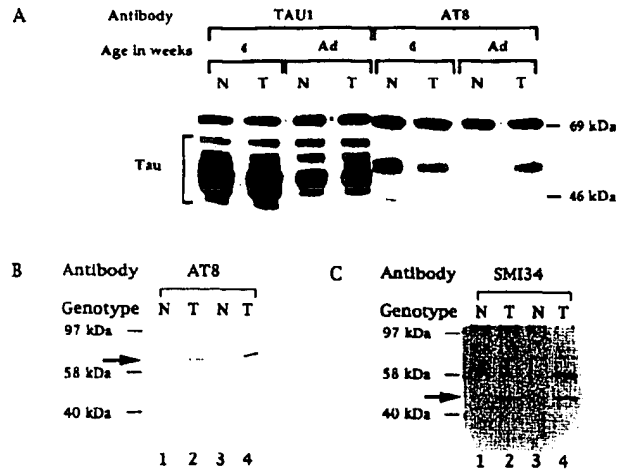


FIG. 3. Expression of tau and neurofilament proteins in *Mos*-transgenic mice. Whole brain (A) or thalamus (B and C) lysates (50 μg/lane) from 4-week-old and adult (Ad) normal (N) and transgenic (T) littermates were analyzed by immunoblotting using monoclonal anti-tau antibodies TAU1 and AT8 (panels A and B) or antineurofilament antibody SMI34 (panel C) as indicated. The samples in panels B and C are from adult mice. The band comigrating with the 69 kDa marker in panel A is an nonspecific chemoluminescence-related background band that is detected also in the absence of specific primary antibody. Immunoblots in panels B and C were developed using the PAP technique. Arrows indicate tau protein species detected by AT8 at elevated levels in transgenic mice (panels A and B) and the novel neurofilament-related protein detected by SMI34 in transgenic mice in panel C.

AT8, which requires serine 202 to be phosphorylated to generate its epitope (2). The two antibodies are, therefore, complementary, as dephosphorylation of the AT8 epitope allows detection by TAU1 and vice versa (2). TAU1 identifies similar bands in both normal and transgenic mice of both ages (Fig. 3A). The change in the electrophoretic mobilities towards higher molecular weight isoforms in older mice is consistent with published reports (23). In contrast, AT8 identifies one predominant band larger in size than the bulk of tau proteins detected by TAU1 in 4-week-old normal and transgenic mice, possibly with a higher signal from the normal mice. In adult mice, this band is detected more strongly in transgenic mice (Fig. 3A), indicating an increase of tau phosphorylation at the AT8 epitope. Analysis of tau proteins with the AT8 antibody in extracts prepared from thalamus, a region severely affected in the *Mos*-transgenic brain (40), yielded similar results (Fig. 3B), demonstrating that neuronal loss in this brain area correlates with an increase of tau phosphorylation at the AT8 epitope.

We also examined possible differences in the expression of neurofilament proteins using a panel of monoclonal antibodies. Representative results obtained with SMI34, which recognizes phosphorylated neurofilament proteins (51), are shown in Fig. 3C. Whereas no significant difference in the phosphorylation of NF-H, NF-M (not shown), and NF-L was detectable in the *Mos*-transgenic brain, we observed a novel protein species of 45–50 kDa in transgenic brain samples. The simplest interpretation of this result is that this protein is a degradation product of phosphorylated neurofilament proteins because it was labeled by all of the monoclonal antibodies used.

Additional experiments aimed at investigating expression and/or status of phosphorylation of microtubule-associated proteins MAP1B and MAP2 a, b, and c did not reveal alterations in protein extracts from brains of *Mos*-transgenic mice (data not shown).

To complement the results obtained by immunoblotting, we performed immunohistochemistry on sections of the normal and *Mos*-transgenic brain. As reported previously (40) transgenic mice exhibited a strong gliosis with numerous GFAP-positive cells in many areas of the brain. These GFAP-positive cells were much less numerous or absent in control animals. In the forebrain of transgenic mice, a strong GFAP-immunoreactivity was observed in the hippocampus (Fig. 4A and B), in many neocortical areas, in the striatum, in the thalamus, and in the corpus callosum. A strong GFAP-immunoreactivity was also detected in the white matter and the cortex of the cerebellum, with a strong labeling of Bergmann glia in the latter. Some astrocytes showed an atypical shape, with irregular and tortuous extensions, or exhibited an extended and balloned cytoplasm.

Staining brain sections for tau with antibodies TAU1 or B19 yielded strong labeling of white matter tracts and in the neuropil of gray matter, whereas only a very weak tau immunoreactivity was detected in perikarya and dendrites. The distribution and intensity of tau immunoreactivity was similar in transgenic and control animals (Fig. 4C and D) and was not markedly affected by pretreatment of the sections with alkaline phosphatase, a procedure that

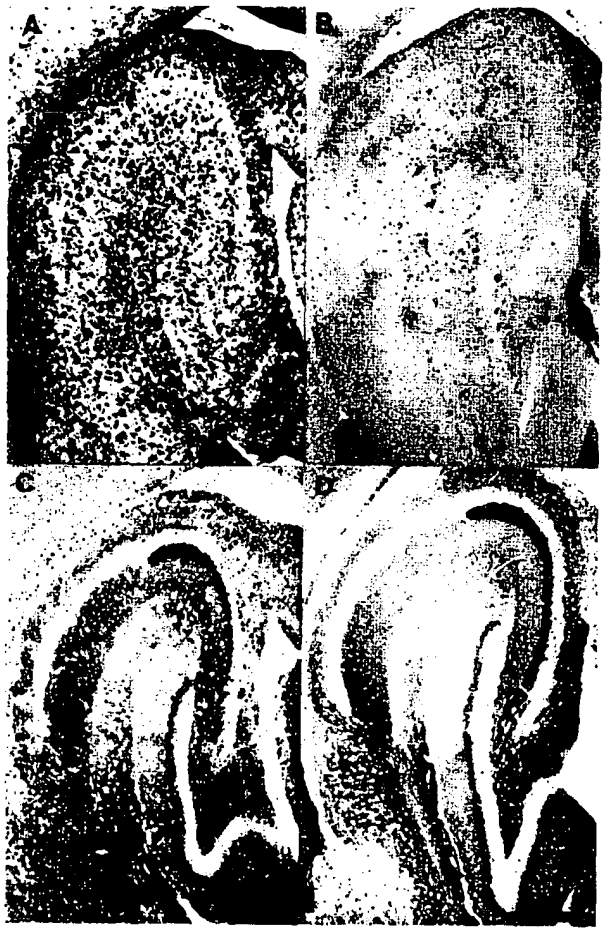


FIG. 4. Immunolabeling (PAP technique) of adjacent sections of the hippocampus (methacarn fixation) from transgenic (A and C) and control (B and D) mice with the anti-GFAP antibody (A and B) and the B19 anti-tau antibody (C and D). A strong gliosis is apparent in the hippocampus of the transgenic animal (A) and is associated with the presence of many small tau-positive cells, predominantly in white matter areas (C). Scale bar: 150 μm.

strongly increases labeling of human neurofibrillary lesions by TAU1 (not shown). In addition to the neuronal labeling, small cells, mainly found in white matter areas exhibited a cytoplasmic tau immunoreactivity in both the normal and transgenic brain. However, in transgenic animals, these cells were much more abundant and their distribution paralleled the distribution of GFAP-positive glial cells (Fig. 4C and D). In contrast to the results obtained by immunoblotting, antibody AT8 directed against phosphorylated tau did not yield significant tissue labeling, either in control or in the transgenic mouse brain sections. As a control we included human brain sections containing neurofibrillary tangles, which were strongly labeled (not shown).

To investigate which cells were responsible for the induction of vimentin expression in the transgenic brain (Fig. 2B), we stained transgenic and control sections with polyclonal and monoclonal antibodies against vimentin. These experiments revealed a strong vimentin immunoreactivity of many astrocytes in the *Mos*-transgenic brain, with only a few astrocytes positive in the normal brain (Fig. 5A and B). However, in the transgenic brain, more astrocytes are positive for GFAP than for vimentin. For example, in the cortex, the number of GFAP-positive cells by far exceeds the number of vimentin-positive cells. In addition to astrocytes, smooth muscle cells in the walls of blood vessels were also labeled by the antivimentin antibody.

Finally, an antibody to β/A4 amyloid did not yield significant labeling in transgenic or control animals (not shown) and antibodies against MAP2 or neurofilament (SMI31), while showing the expected dendritic and axonal labeling, respectively, did not reveal differences between the normal and transgenic brain (not shown).

DISCUSSION

We have previously demonstrated that overexpression of the protein-serine/threonine kinase Mos in the brain of transgenic mice leads to severe brain pathology including vacuolation of the neuropil, neuronal, and axonal degeneration and gliosis (39,40). These observations gained renewed interest in the light of two recently published results. First, it was shown that the Mos protein kinase binds to microtubules in vivo (57), and secondly, Mos activates MAPK, presumably by phosphorylating and thereby activating MAPKK (31,38,46). Because MAPK, among other recently identified proline-directed kinases, has been implicated in the aberrant phosphorylation of cytoskeletal proteins found in neurodegenerative conditions (2), we thought to examine the expression and/or phosphorylation of cytoskeletal proteins that might be involved in the neuropathology of *Mos*-transgenic mice.

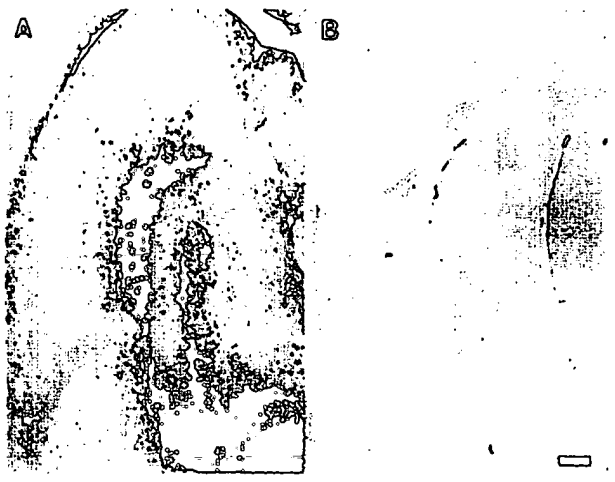


FIG. 5. Immunolabeling (PAP technique) of adjacent sections of the hippocampus (methacarn fixation) from transgenic (A) and control (B) mice with the antivimentin antibody. Many astrocytes are vimentin positive in the transgenic animal (A) but not in the control (B). Scale bar: 100 μm.

The analysis of glial intermediate filament proteins revealed a strong increase of both GFAP and vimentin during postnatal development of the transgenic brain. This increase occurred in parallel to the postnatal onset of transgene expression and is consistent with the previously observed development of gliosis in these animals (40). Of particular interest in this context is the pattern of vimentin expression. During the early postnatal period, vimentin expression in the transgenic brain showed the developmental decrease expected from published results obtained in normal mice (13,14). Thereafter, beginning at 3–4 weeks of age, vimentin expression dramatically increased and during certain developmental stages (4–6 weeks) we observed, in addition to 55 kDa vimentin, a 50 kDa protein reacting with the monoclonal antivimentin antibody. We have not analyzed this protein further, but its appearance is remeniscent of the detection of a proteolytic vimentin fragment in *Mos*-transformed cells (47). In the *Mos*-transgenic brain, the postnatal increase in vimentin levels was due to expression in a subset of GFAP positive astrocytes. This reinduction of vimentin expression in mature astrocytes might be due to the action of the protooncogen *Mos*, as vimentin expression in neoplastic astrocytes has been documented (19,45,55). However, whereas atypical, perhaps preneoplastic, astrocytes are frequently observed in the *Mos*-transgenic brain, frank astrocytomas are rare (39,40). On the other hand, vimentin expression has been demonstrated in reactive astrocytes in rats with experimental allergic encephalomyelitis (8).

In addition to vimentin we observed tau-positive cells in the white matter of *Mos*-transgenic brain. The distribution of these cells paralleled that of GFAP-positive cells, suggesting that tau, too, was expressed in astrocytes. A tau-immunoreactivity in astrocytes has been described previously (12,35). Attempts to clarify whether it was these white matter cells rather than neurons that expressed tau containing phosphorylation-sensitive AT8-reactive epitopes by immunohistochemistry were unsuccessful (see below).

The neuronal cytoskeletal proteins most commonly affected in neurodegenerative diseases are the microtubule-associated protein tau and the neurofilament proteins (29,37,48,49,52). Analysis of tau phosphorylation in *Mos*-transgenic mouse brain by a panel of monoclonal antibodies recognizing phosphorylated epitopes on tau revealed increased phosphorylation on one of these epitopes. This epitope contains serines 199 and 202 and is recognized by the AT8 antibody. Other antibodies, such as 8D8, which recognize different phosphorylated epitopes on tau, did not reveal differences between normal and transgenic mice (not shown). Tau phosphorylation on the AT8 epitope was detected by immunoblot analysis of protein extracts from whole brain or thalamus, an area strongly affected in the transgenic brain. However, we were unable to clarify which cell type might contain hyperphosphorylated tau due to the lack of AT8 staining in histological sections. An absence of AT8-immunoreactivity in the adult rat brain by immunohistochemistry on methacarn-fixed tissue has been observed (6), although the detection of an AT8-immunoreactivity by immunoblotting in adult rat brain has been reported (27). Factors affecting the detection of phosphorylated tau in tissue sections might be the fixation conditions, the absolute level of the phosphorylated species, the postmortem action of protein phosphatases or the masking of the phosphorylated epitope in sections but not on blots. The latter has been observed with another microtubule-associated protein, MAP1B (17).

Several candidates of proline directed protein-serine/threonine kinases, including MAPK, have emerged capable of phosphory-

lating tau. Given the fact that Mos activates the MAPK signal transduction pathway in several in vivo and in vitro systems, we investigated a possible activation of MAPK in transgenic brain extracts. However, in preliminary experiments we did not detect such an activation of MAPK (not shown). Other candidates of proline-directed protein kinases that might be responsible for Mos-induced phosphorylation of tau on serines 199 and or 202 include other MAPK related kinases, cdk5/tau protein kinase II (21,26,30), and glycogen synthase kinase 3β/tau protein kinase I (25). Whether any of these kinases is, indeed, involved or whether Mos can phosphorylate tau directly remains to be determined.

In addition to tau, neurofilament proteins are altered in *Mos*-transgenic mice. We have previously shown by immunohistochemistry that phosphorylated neurofilament epitopes, normally present exclusively in axons, can be detected in the perikarya of transgenic neurons (39,40). The immunoblot analysis carried out here did not, however, reveal detectable differences between normal and transgenic littermates. This is probably due to the relative insensitivity of the immunoblot analysis of whole brain extracts. On the other hand, we were able to detect a novel 45–50 kDa protein species reacting with several antibodies reactive with neurofilament heavy chain. It is conceivable that this protein is a degradation product of the neurofilament heavy chain. As mentioned above, a similar mechanism might account for the 50 kDa vimentin-related protein found in transgenic brain, implying that both glial and neuronal intermediate filaments are subject to this alteration. Interestingly, evidence for keratin proteolysis has been obtained in hepatocytes under conditions where the intermediate filament is hyperphosphorylated and aggregates into cytoplasmic inclusions termed Malory bodies (44). Thus, the possible detection of neurofilament and vimentin degradation in *Mos*-transgenic brain might be a sensitive indicator of intermediate filament abnormalities.

To our knowledge c-*Mos*- and v-*Mos*-transgenic mice are unique in demonstrating that overexpression in the brain of a protein-serine/threonine kinase implicated in the MAPK signal transduction pathway can trigger some but not all of the symptoms associated with human progressive neurodegenerative diseases. Whereas there is extensive and progressive neuronal loss and gliosis, neurofibrillary tangles, paired helical filaments, and amyloid plaques have not been observed. Likewise, tau appears to be hyperphosphorylated to some extent but only on one of the sites found in PHF-tau in Alzheimer's disease. It is conceivable that individual neurodegenerative diseases, while displaying unique and specific features, also share common mechanisms leading to neuronal loss. It is our hope that several different animal models such as *Mos*-transgenic mice, each displaying a different subset of neurodegenerative features, might contribute to a distinction between specific and common mechanisms in neurodegeneration.

ACKNOWLEDGEMENTS

We are grateful to Innogenetics, Ghent, Belgium for providing the monoclonal anti-tau antibody AT8. This study was supported by The Wellcome Trust, MRC, the UK Alzheimer's Disease Society, and by a grant from the European Neuroscience Program.

REFERENCES

1. Berling, B.; Wille, H.; Röll, B.; Mandelkow, E.-M.; Garner, C.; Mandelkow, E. Phosphorylation of microtubule-associated proteins MAP2a,b and MAP2c at Ser136 by proline-directed kinases in vivo and in vitro. Eur. J. Cell Biol. 64:120–130; 1994.
2. Biernat, J.; Mandelkow, E.-M.; Schröter, C.; Lichtenberg-Kraag, B.; Steiner, B.; Berling, B.; Meyer, H.; Mercken, M.; Vandermeeren, A.; Goedert, M.; Mandelkow, E. The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. EMBO J. 11:1593–1597; 1992.
3. Binder, L. I.; Frankfurter, A.; Rebhun, L. I. The distribution of tau in the mammalian central nervous system. J. Cell Biol. 101:1371–1378; 1985.
4. Brion, J.-P.; Guilleminot, J.; Couchie, D.; Nunez, J. Both adult and juvenile tau microtubule-associated proteins are axon specific in the developing and adult rat cerebellum. Neuroscience 25:139–146; 1988.
5. Brion, J.-P.; Hanger, D. P.; Bruce, M. T.; Couck, A. M.; Flament-Durand, J.; Anderton, B. H. Tau in Alzheimer neurofibrillary tangles: *N*- and *C*-terminal regions are differentially associated with paired helical filaments and the location of a putative abnormal phosphorylation site. Biochem. J. 273:127–133; 1991.
6. Brion, J.-P.; Octave, J. N.; Couck, A. M. Distribution of the phosphorylated microtubule-associated protein tau in developing cortical neurons. Neuroscience 63:895–909; 1994.
7. Brion, J.-P.; Smith, C.; Couck, A.-M.; Gallo, J.-M.; Anderton, B. H. Developmental changes in τ phosphorylation: fetal τ is transiently phosphorylated in a manner similar to paired helical filament-τ characteristic of Alzheimer's disease. J. Neurochem. 61:2071–2080; 1993.
8. Cammer, W.; Tansey, F. A.; Brosnan, C. F. Gliosis in the spinal cords of rats with experimental allergic encephalomyelitis: Immunostaining of carbonic anhydrase and vimentin in reactive astrocytes. Glia 2:223–230; 1989.
9. Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159; 1987.
10. Coleman, M. P.; Anderton, B. H. Phosphate-dependent monoclonal antibodies to neurofilaments and Alzheimer neurofibrillary tangles recognize a synthetic phosphopeptide. J. Neurochem. 54:1548–1555; 1990.
11. Colledge, W. H.; Carlton, M. B. L.; Udy, G. B.; Evans, M. J. Disruption of c-*mos* causes parthenogenetic development of unfertilized mouse eggs. Nature 370:65–68; 1994.
12. Couchie, D.; Fages, C.; Bridoux, A. M.; Rolland, B.; Tardy, M.; Nunez, J. Microtubule-associated proteins and in vitro astrocyte differentiation. J. Cell Biol. 101:2095–2103; 1985.
13. Dahl, D. The vimentin-GFA protein transition in rat neuroglia cytoskeleton occurs at the time of myelination. J. Neurosci. Res. 6:741–748; 1981.
14. Dahl, D.; Rueger, D. C.; Bignami, A.; Weber, K.; Osborn, M. Vimentin, the 57,000 molecular weight protein of fibroblast filaments, is the major cytoskeletal component in immature glia. Eur. J. Cell Biol. 24:191–196; 1981.
15. Drewes, G.; Lichtenberg-Kraag, B.; Döring, F.; Mandelkow, E.-M.; Biernat, J.; Goris, J.; Dorée, M.; Mandelkow, E. Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. EMBO J. 11:2131–2138; 1992.
16. Freeman, R. S.; Kanki, J. P.; Ballantyne, S. M.; Pickham, K. M.; Donoghue, D. J. Effects of the v-*mos* oncogene on *Xenopus* development: Meiotic induction in oocytes and mitotic arrest in cleaving embryos. J. Cell Biol. 111:533–541; 1990.
17. Harrison, L.; Cheetham, M. E.; Calvert, R. A. Investigations of the changes in neuronal distribution and phosphorylation state of MAP1X during development. Dev. Neurosci. 15:68–76; 1993.
18. Hashimoto, N.; Watanabe, N.; Furuta, Y.; Tamemoto, H.; Sagata, N.; Yokoyama, M.; Okazaki, K.; Nagayoshi, M.; Takeda, N.; Ikawa, Y.; Aizawa, S. Parthenogenetic activation of oocytes in c-*mos*-deficient mice. Nature 370:68–71; 1994.
19. Herpers, M. J.; Ramaekers, F. C.; Aldeweireldt, J.; Moesker, O.; Slooff, J. Co-expression of glial fibrillary acidic protein- and vimentin-type intermediate filaments in human astrocytomas. Acta Neuropathol. Berl. 70:333–339; 1986.
20. Johnstone, A.; Thorpe, R. Immunochemistry in practice. Oxford, UK: Blackwell Scientific Publications; 1982.
21. Kobayashi, S.; Ishiguro, K.; Omori, A.; Takamatsu, M.; Arioka, M.;

Imahori, K.; Uchida, T. A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30 kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. FEBS Lett. 335: 171–175; 1993.
22. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685; 1970.
23. Larcher, J. C.; Boucher, D.; Ginzburg, I.; Gros, F.; Denoulet, P. Heterogeneity of tau proteins during mouse brain development and differentiation of cultured neurons. Dev. Biol. 154:195–204; 1992.
24. Lichtenberg-Kraag, B.; Mandelkow, E.-M.; Biernat, J.; Steiner, B.; Schröter, C.; Gustke, N.; Meyer, H. E.; Mandelkow, E. Phosphorylation-dependent epitopes of neurofilament antibodies on tau protein and relationship with Alzheimer tau. Proc. Natl. Acad. Sci. USA 89:5384–5388; 1992.
25. Lovestone, S.; Reynolds, C. H.; Latimer, D.; Davis, D. R.; Anderton, B. H.; Gallo, J.-M.; Hanger, D.; Mulot, S.; Marquardt, B.; Stubel, S.; Woodgett, J. R.; Miller, C. C. J. Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. Curr. Biol. 4:1077–1086; 1994.
26. Lu, Q.; Soria, J. P.; Wood, J. G. $p44^{mpk}$ MAP kinase induces Alzheimer type alterations in tau function and in primary hippocampal neurons. J. Neurosci. Res. 35:439–444; 1993.
27. Matsuo, E. S.; Shin, R.-W.; Billingsley, M. L.; Van DeVoorde, A.; O'Connor, M.; Trojanowski, J. Q.; Lee, V. M.-Y. Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. Neuron 13:989–1002; 1994.
28. Maxwell, S. A.; Arlinghaus, R. B. Serine kinase activity associated with Moloney murine sarcoma virus-124-encoded $p37^{mos}$. Virology 143:321–333; 1985.
29. Mizusawa, H.; Matsumoto, S.; Yen, S. H.; Hirano, A.; Rojas Corona, R. R.; Donnenfeld, H. Focal accumulation of phosphorylated neurofilaments within anterior horn cell in familial amyotrophic lateral sclerosis. Acta Neuropathol. Berl. 79:37–43; 1989.
30. Mohit, A. A.; Martin, J. H.; Miller, C. A. $p49^{SPK12}$ kinase: A novel MAP kinase expressed in a subset of neurons in the human nervous system. Neuron 14:67–78; 1995.
31. Nebreda, A. R.; Hill, C.; Gomez, N.; Cohen, P.; Hunt, T. The protein kinase *mos* activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cells in vivo. FEBS Lett. 333:183–187; 1993.
32. Nixon, R. A. The regulation of neurofilament protein dynamics by phosphorylation: Clues to neurofibrillary pathobiology. Brain Pathol. 3:29–38; 1993.
33. O'Keefe, S. J.; Wolfes, H.; Kiessling, A. A.; Cooper, G. M. Microinjection of antisense *c-mos* oligonucleotides prevents meiosis II in the maturing mouse egg. Proc. Natl. Acad. Sci. USA 86:7038–7042; 1989.
34. Oskarsson, M.; McClements, W. L.; Blair, D. G.; Maizel, J. V.; Vande Woude, G. F. Properties of a normal mouse cell DNA sequence (sarc) homologous to the src sequence of Moloney sarcoma virus. Science 207:1222–1224; 1980.
35. Papasozomenos, S. C.; Binder, L. I. Phosphorylation determines two distinct species of tau in the central nervous system. Cell Motil. Cytoskel. 8:210–226; 1987.
36. Paules, R. S.; Buccione, R.; Moschel, R. C.; Vande Woude, G. F.; Eppig, J. J. Mouse *Mos* protooncogene product is present and functions during oogenesis. Proc. Natl. Acad. Sci. USA 86:5395–5399; 1989.
37. Pollanen, M. S.; Dickson, D. W.; Bergeron, C. Pathology and biology of the Lewy body. J. Neuropathol. Exp. Neurol. 52:183–191; 1993.
38. Posada, J.; Yew, N.; Ahn, N. G.; Vande Woude, G. F.; Cooper, J. A. Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase in vitro. Mol. Cell. Biol. 13:2546–2553; 1993.
39. Propst, F.; Cork, L. C.; Kovatch, R. M.; Kasenally, A. B.; Wallace, R.; Rosenberg, M. P. Progressive hind limb paralysis in mice carrying a *v-Mos* transgene. J. Neuropathol. Exp. Neurol. 51:499–505; 1992.
40. Propst, F.; Rosenberg, M. P.; Cork, L. C.; Kovatch, R. M.; Rauch, S.; Westphal, H.; Khillan, J.; Schulz, N. T.; Vande Woude, G. F.; Newmann, P. E. Neuropathological changes in transgenic mice carrying copies of a transcriptionally activated *Mos* protooncogene. Proc. Natl. Acad. Sci. USA 87:9703–9707; 1990.
41. Roy, L. M.; Singh, B.; Gautier, J.; Arlinghaus, R. B.; Nordeen, S. K.; Maller, J. L. The cyclin B2 component of MPF is a substrate for the *c-mos*^{xe} proto-oncogene product. Cell 61:825–831; 1990.
42. Sagata, N.; Oskarsson, M.; Copeland, T.; Brumbaugh, J.; Vande Woude, G. F. Function of *c-mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. Nature 335:519–525; 1988.
43. Sagata, N.; Watanabe, N.; Vande Woude, G. F.; Ikawa, Y. The *c-mos* proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. Nature 342:512–518; 1989.
44. Salmhofer, H.; Rainer, I.; Zatloukal, K.; Denk, H. Posttranslational events involved in griseofulvin-induced keratin cytoskeleton alterations. Hepatology 20:731–740; 1994.
45. Schiffer, D.; Giordana, M. T.; Mauro, A.; Migheli, A.; Germano, I.; Giaccone, G. Immunohistochemical demonstration of vimentin in human cerebral tumors. Acta Neuropathol. Berl. 70:209–219; 1986.
46. Shibuya, E. K.; Ruderman, J. V. Mos induces the in vitro activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. Mol. Biol. Cell 4:781–790; 1993.
47. Singh, B.; Arlinghaus, R. B. Vimentin phosphorylation by $p37^{mos}$ protein kinase in vitro and generation of a 50-kDa cleavage product in *v-mos*-transformed cells. Virology 173:144–156; 1989.
48. Smith, C.; Anderton, B. H. The molecular pathology of Alzheimer's disease: Are we any closer to understanding the neurodegenerative process. Neuropathol. Appl. Neurobiol. 20:322–338; 1994.
49. Sobue, G.; Hashizume, Y.; Yasuda, T.; Mukai, E.; Kumagi, T.; Mitsuma, T.; Trojanowski, J. Q. Phosphorylated high molecular weight neurofilament protein in lower motor neurons in amyotrophic lateral sclerosis and other neurodegenerative diseases involving ventral horn cells. Acta Neuropathol. Berl. 79:402–408; 1990.
50. Southern, E. M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517; 1975.
51. Sternberger, L. A.; Sternberger, N. H. Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments in situ. Proc. Natl. Acad. Sci. USA 80:6126–6130; 1983.
52. Trojanowski, J. Q.; Schmidt, M. L.; Ryong-Woon, S.; Bramblett, G. T.; Rao, D.; Lee, V. M. Y. Altered tau and neurofilament proteins in neurodegenerative diseases: Diagnostic implications for Alzheimer's disease and Lewy body dementias. Brain Pathol. 3:45–54; 1993.
53. Yew, N.; Mellini, M.; Vande Woude, G. F. Meiotic initiation by the *mos* protein in *Xenopus*. Nature 355:649–652; 1992.
54. Yew, N.; Oskarsson, M.; Daar, I.; Blair, D. G.; Vande Woude, G. F. *mos* gene transforming efficiencies correlate with oocyte maturation and cytostatic factor activities. Mol. Cell. Biol. 11:604–610; 1991.
55. Yung, W. K.; Luna, M.; Borit, A. Vimentin and glial fibrillary acidic protein in human brain tumors. J. Neurooncol. 3:35–38; 1985.
56. Zhao, X.; Batten, B.; Singh, B.; Arlinghaus, R. B. Requirement of the *c-mos* protein kinase for murine meiotic maturation. Oncogene 5: 1727–1730; 1990.
57. Zhou, R.; Rulong, S.; Pinto da Silva, P.; Vande Woude, G. F. In vitro and in vivo characterization of $pp39^{mos}$ association with tubulin. Cell Growth Diff. 2:257–265; 1991.

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FEBS Letters 1995 Feb 20, 360(1):5-9

FEBS Letters 1994 March 14, 341(1):94-98

J. of Neurochem., 1995 April 64(4):1759-68

Neurochemistry International 1995 March 26(3):205-15

Biochem J., 1995 Aug 1, 309(Pt 3):941-9

Neurobiol of Aging 1996 March-April, 17(2):235-41

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(703) 308-0056

Biochem. J. (1995) 309, 941–949 (Printed in Great Britain)

The phosphorylation state of the microtubule-associated protein tau as affected by glutamate, colchicine and β-amyloid in primary rat cortical neuronal cultures

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The effects of the excitatory amino acid glutamate, the microtubule destabilizing agent colchicine, and β_{25-35}-amyloid peptide on the phosphorylation state of tau were studied in rat cortical neurons in primary culture. Using immunocytochemistry and Western-blot analysis, we demonstrated that a proportion of tau in these cultures is normally highly phosphorylated, but most of this tau fraction is dephosphorylated after treatment of the cultures with glutamate or colchicine, but not with β-amyloid; the glutamate- and colchicine-induced changes in tau phosphorylation commenced before cell death, as assessed by release of lactate dehydrogenase. Dephosphorylation of tau was readily revealed by using the monoclonal antibodies Tau.1 and AT8, which have phosphate-sensitive epitopes that both centre around serine-199 and -202 (numbering of the largest tau isoform). On Western blots and by immunocytochemistry, AT8 labelling strongly decreased after glutamate and colchicine treatments, whereas Tau.1 staining was more intense. Neurofilament monoclonal antibodies, including RT97, 8D8, SMI31 and SMI310, all additionally known to recognize tau in a phosphorylation-dependent manner, also demonstrated that glutamate and colchicine treatments of the cultures induced a dephosphorylation of tau. We also showed immunocytochemically that there is an increase in tau immunoreactivity in neuronal perikarya in response to glutamate and colchicine treatment, and this occurs concomitantly with the dephosphorylation of tau. Treatment of the primary rat cortical neuronal cultures with β_{25-35}-amyloid peptide, under conditions which induce neuronal degeneration, did not induce a change in tau phosphorylation, and failed to act synergistically with glutamate to produce an increase in dephosphorylation of tau over that produced by glutamate treatment alone. These findings demonstrate that glutamate and colchicine induce tau dephosphorylation, as opposed to increased tau phosphorylation, which would be more indicative of Alzheimer-type neurodegeneration.

INTRODUCTION

The molecular events underlying neurodegeneration of Alzheimer's disease are not established, but it has been suggested that an excitotoxic mechanism mediated by excessive extracellular glutamate may be a contributory factor, and more recently the neurotoxicity of aggregated β-amyloid has received much attention (for review see [1]). In support of an excitotoxic mechanism, Mattson and colleagues have reported that glutamate and β-amyloid act synergistically to cause neuronal death *in vitro* and that this cell death is preceded by an increase in perikaryal tau immunoreactivity, which is also produced by treatment of neurons with colchicine [2,3]; these changes in perikaryal tau were suggested to mimic those observed in neurofibrillary tangle-bearing neurons in Alzheimer's disease [2]. Since at least many degenerating neurons in the Alzheimer's-disease brain exhibit a characteristic cytoskeletal pathology, particularly hyperphosphorylated paired helical filament (PHF) tau, we set out to investigate whether in cultured neurons neurotoxicity induced by glutamate, colchicine and β-amyloid produced changes in the phosphorylation state of tau that may parallel those occurring in Alzheimer's disease.

The microtubule-associated phosphoprotein tau is a major constituent of PHF, which constitute neurofibrillary tangles, a pathological hallmark of Alzheimer's disease [4,5]. PHF-tau differs from tau isolated from normal adult human brain in that it is resolved on SDS/PAGE as three to four bands of apparent molecular mass 60–68 kDa, instead of the six or more bands of apparent molecular masses between 55 and 62 kDa that are characteristic of normal adult brain tau [6–10]; PHF-tau can be induced to migrate like normal tau by treatment with alkaline phosphatase, suggesting that it is excessively phosphorylated [9–11]. PHF-tau has also been shown to be hyperphosphorylated through the use of a number of monoclonal antibodies that recognize specific phosphorylated epitopes and discriminate normal adult brain tau from PHF-tau [12,13]. By contrast, tau extracted from foetal brain comprises a single isoform that corresponds to the shortest adult brain tau species, and a fraction of this foetal tau is in an elevated state of phosphorylation, resembling the phosphorylation state of PHF-tau [14–16].

The majority of investigations of immunocytochemical changes in tau in response to excitotoxic levels of glutamate and other agents have employed only a limited number of antibodies to tau, and have not used the m noclonal antibodies that have

Abbreviations used: PHF, paired helical filament; HBSS, Hanks balanced salt-solution; LDH, lactate dehydrogenase; TBS, Tris-buffered saline.

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more recently been demonstrated to discriminate control adult brain tau from the more highly phosphorylated PHF-tau. It has only recently been realized that the phosphorylation state f a proportion of foetal tau is elevated compared with control adult brain tau, and that this fraction of foetal tau already resembles PHF-tau, as assessed by application of discriminatory antibodies [14–18]. In the present study we have used a panel of such monoclonal antibodies and taken account of the known higher level of phosphorylation of some of the foetal tau to re-examine the effects of toxic levels of glutamate, colchicine and β-amyloid on tau phosphorylation in rat cortical neurons in primary culture.

We report here that a proportion of tau in cultured rat cortical neurons normally has a higher level of phosphorylation than control adult brain tau, and is therefore similar to foetal tau *in vivo* and to PHF-tau from Alzheimer's-disease brain, and that dephosphorylation of this tau occurs after treatment with toxic levels of glutamate or colchicine, but before cell death, as assessed by release of lactate dehydrogenase. Concomitant with dephosphorylation, perikaryal tau immunoreactivity is increased, as assessed by immunocytochemical analyses of the cultures. These changes in tau phosphorylation in response to glutamate and colchicine treatment are opposite to what would be expected if they triggered the formation of PHF. Furthermore, we were unable to induce any changes in tau phosphorylation by exposing cultures to toxic doses of β-amyloid peptide in either the absence or the presence of glutamate, until there was obvious cell death, when the loss of tau immunoreactivity was most likely due to non-specific effects, e.g. release of lysosomal hydrolases.

MATERIALS AND METHODS

Antibodies

The preparation of the affinity-purified polyclonal antibody to human tau, 8073, and polyclonal antibody to bovine tau, B19, that recognize all tau isoforms independently of their phosphorylation states, have previously been described [8,19]. The mouse monoclonal antibody, Tau.1, that recognizes a dephosphorylated epitope in normal tau [20] was generously given by Dr. L. I. Binder (University of Alabama, Birmingham, AL, U.S.A.). The monoclonal antibody, AT8 [21], that recognizes a phosphorylated epitope (Ser-202) on PHF-tau and that corresponds to the Tau.1 epitope when unphosphorylated (Ser-199 and Ser-202) was a gift from Innogenetics Ltd., Zwijndrecht, Belgium. Antiserum TP70, raised to a synthetic C-terminal tau peptide, has been previously described [12]. The TP007 antiserum was raised to a synthetic peptide corresponding to the 16 most N-terminal residues of human tau that are present in all tau isoforms. RT97, 8D8, SMI31 and SMI310 are monoclonal antibodies that recognize phosphorylated neurofilament and PHF-tau [12,13,22–24].

Primary neuronal culture

Embryonic-rat (E17) cortices were dissected into Hanks balanced salt solution (HBSS; Gibco, Paisley, Scotland, U.K.), cleaned of meninges and chopped. HBSS was removed by aspiration and the tissue was incubated in HBSS containing 0.05% (w/v) trypsin (Sigma, Poole, Dorset, U.K.) at 37 °C for 15 min. The trypsin solution was then removed by aspiration and replaced with HBSS containing 8 mM $MgCl_2$, 0.002% (w/v) DNase, 10 mM Hepes and 5% (v/v) foetal-calf serum. The tissue was triturated in 2.5 ml of the same solution with a fire-polished glass Pasteur pipette. The cell suspension was diluted to 1×10^6 cells/ml in glutamine-free tissue-culture medium that consisted of Dulbecco's modified Eagle's medium mixed with Ham's F12 medium [DMEM:F12, 4:1 (v/v); Sigma] containing 100 μg/ml transferrin, 60 μM putrescine, 5 μg/ml insulin, 20 nM progesterone, 30 nM sodium selenite, 50 units/ml penicillin, 50 μg/ml streptomycin and 2% (v/v) heat-inactivated horse serum (all from Sigma) and plated into 25 cm^2 flasks (Marathon, London, U.K.) at a density of 3.2×10^5 cells/cm, or into 35 mm × 10 mm culture dishes (Marathon) at a density of 1.6×10^5 cells/cm that had been precoated with poly-L-lysine (10 μg/ml) and laminin (10 μg/ml) (both Sigma). Half of the tissue-culture medium was replaced after 5–7 days with DMEM:F12 (4:1, v/v) containing the supplements specified above, except without the addition of serum. This low-serum medium was sufficient to prevent n n-neuronal cells proliferating. At 24 h before treatments, medium was fully replaced with the same serum-free media. Glutamate-, colchicine- and β-amyloid-induced degeneration of neuronal cultures was assessed by the release of lactate dehydrogenase (LDH) into the culture medium [25] (see below).

Experimental treatments

After 6, 8 or 10 days in culture, the cells were treated with 1 mM L-glutamate, 1 μM colchicine or 250 nM okadaic acid (Sigma) for up to 6 h, or with 100 μM β_{25-35}-amyloid peptide (BaChem, Torrance, CA, U.S.A.) for up to 24 h. The β_{25-35}-amyloid peptide was stored at room temperature in aqueous solution before addition to culture media; this 'aging' induced aggregation of the peptide, which is known to increase its neurotoxic properties. After treatments, portions of tissue-culture media were centrifuged at 15800 $g_{av.}$ and supernatants were stored at −70 °C for determination of LDH. Cells from tissue-culture flasks were harvested into PBS and pelleted, whereas those in culture dishes were used for immunocytochemical analysis (see below). For total cell-lysate protein preparations, the cell pellet from each flask was resuspended in 0.5 ml of PBS, and a 50 μl portion was added to SDS/PAGE sample buffer [62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue] for electrophoretic analysis (see below), heated for 5 min in a boiling-water bath and centrifuged at 15800 $g_{av.}$ for a further 5 min. The remaining cell suspension was centrifuged (15800 $g_{av.}$, 1 min), and the pellet was resuspended in 40–100 μl of Mes/NaCl buffer (100 mM Mes, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 M NaCl, 2 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride pH 6.5) and heated for 5 min in a boiling-water bath. The preparation was then centrifuged at 15800 $g_{av.}$ for 25 min. The supernatant containing the heat-stable fraction enriched in tau was retained; SDS/PAGE sample buffer was added, followed by a further 5 min incubation in a boiling-water bath.

For alkaline phosphatase treatment, cultured neurons were harvested into PBS, pelleted and resuspended in 100 μl of Mes/NaCl buffer, heated for 5 min in a boiling-water bath and centrifuged at 15800 $g_{av.}$ for 25 min. The supernatant, enriched in tau, was removed and dialysed against 50 mM Tris, pH 8.3, containing 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM phenylmethanesulphonyl fluoride, 1 mg/ml pepstatin, 0.2 mg/ml aprotinin, 0.5 mg/ml leupeptin and 100 mM benzamidine (alkaline phosphatase reaction buffer). Alkaline phosphatase from calf intestinal mucosa (Boehringer Mannheim, Lewes, Sussex, U.K.) was added t portions of dialysed protein to give a final concentration of 400 units/ml and incubated vernight at 37 °C; for c ntrol samples, enzyme was omitted. After incubation, samples were heated in a boiling-water bath f r 5 min, and SDS/PAGE sample buffer was added, f llowed by a further 5 min incubation peri d in a b iling-water bath.

Determination of neurotoxicity

LDH was assessed in culture media by using a LDH isoenzyme assay kit (Sigma) as an indication of neuronal cell damage [25]. Samples of media in incubation buffer were mixed with Cellustain reagent [10 parts of NAD^+, 85 parts of lithium L(+)-lactate, 5 parts of Nitro Blue Tetrazolium and 0.1 part of phenazine methosulphate] and incubated at room temperature in the dark for up to 30 min. The A_{550} of the resulting colour was measured. The change in A_{550} measured in cultures treated with glutamate for 24 h, in which all neurons were dead, was taken as 100% LDH release. The means of six measurements from each treatment group were compared with the control group by Student's t test.

SDS/PAGE and Western blotting

Samples of total lysates of cultured cells and heat-stable preparations were separated on SDS/10% (w/v)-polyacrylamide gels in a discontinuous buffer system [26]. Separated proteins were then electrophoretically transferred on to nitrocellulose (Schleicher and Schuell, 0.45 μm pore size; Anderman, Kingston-on-Thames, Surrey, U.K.), blocked in Tris-buffered saline (TBS; 25 mM Tris, pH 8.0, 140 mM NaCl, 5 mM KCl) containing 0.2% (v/v) Tween 20 and 3% (w/v) dried skimmed milk (TBS-T-M) and incubated overnight in primary antibody diluted in TBS-T-M at 4 °C as described previously [9]. The filter was then washed (3 × 10 min in TBS containing 0.2% Tween 20; TBS-T) and incubated for 1 h in biotinylated donkey anti-rabbit or biotinylated sheep anti-mouse secondary antibody (Amersham International, Amersham, Bucks., U.K.; diluted 1:500 in TBS-T-M), followed by further washing in TBS-T and incubation for 1 h in streptavidin–alkaline phosphatase (Amersham; diluted 1:3000 in TBS-T-M). After final washes in TBS-T, blots were developed by using an alkaline phosphatase conjugate substrate kit (Bio-Rad, Hemel Hempstead, Herts., U.K.). Before separation by SDS/PAGE, the protein content of each sample was determined, by using a dot-blot stained with Coomassie Blue and so that loading on to the electrophoresis gels could be normalized for total protein.

Immunocytochemical labelling of cultures

Cultures were fixed for 20 min with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, washed in the same phosphate buffer and stored at 4 °C in TBS. These tissue-culture samples were labelled by the peroxidase–antiperoxidase (PAP) method. Cultures were first treated for 30 min with TBS containing 0.2% Triton X-100 (TBS-TX), incubated in blocking solution [TBS-TX containing 10% (v/v) normal goat serum], and then treated for 16 h at 20 °C with the primary antibody diluted in TBS-TX containing 1% normal goat serum. The cultures were then incubated with a goat anti-rabbit or goat anti-mouse antibody (Nordic, Turnhout, Belgium; diluted 1:30 in TBS-TX containing 1% normal goat serum) followed by a rabbit or mouse PAP complex (Nordic; diluted 1:300 in TBS-TX containing 1% normal goat serum). The peroxidase activity was revealed by using diaminobenzidine as chromogen.

RESULTS

Glutamate-induced dephosphorylation of tau in primary rat cortical neuronal cultures

Initially, the effects of the excitatory amino acid glutamate on the phosphorylation state of foetal tau were studied in 10-day-old primary rat cortical neuronal cultures. Figure 1 shows Western blots of total cell-lysate proteins from cultures treated with (Figure 1a) glutamate (1 mM) and (Figure 1b) colchicine (1 μM) for up to 6 h and labelled with an affinity-purified polyclonal antibody to human tau, 8073, and the monoclonal antibody, Tau.1, as indicated. As we described previously for foetal tau *in vivo*, the major tau species run either as a broad band or as a closely migrating and poorly resolved doublet of approx. 48 kDa (arrows), that is labelled by the polyclonal antibody, but only the faster-migrating species of the doublet (or portion of the broad band) was labelled by Tau.1 [14]. However, after incubation for 30 min with glutamate, two lower-molecular-mass bands (arrowheads) were recognized by 8073 and Tau.1 (Figure 1a, lanes 3), which became more intense with prolonged glutamate incubation (Figure 1a, lanes 4–6). A similar pattern of tau immunoreactivity was observed after colchicine treatment (Figure 1b), in that the same faster-migrating tau species (arrowheads) became more intensely labelled by 8073 and Tau.1 after incubation with colchicine for 30 min (Figure 1b, lanes 3), and became more intensely labelled with prolonged exposure (Figure 1b, lanes 4–6).

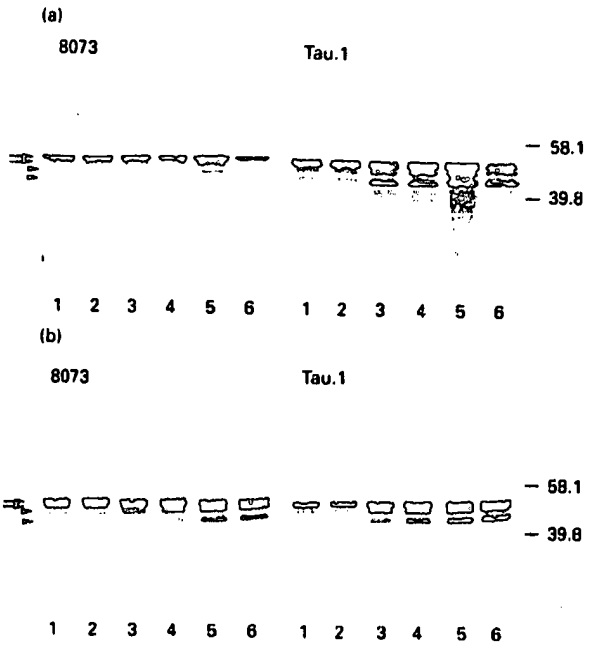

Figure 1 Time-course effect of glutamate and colchicine on tau in primary neuronal cultures

Western blots of total cell-lysate proteins from primary 10-day-old rat cortical neuronal cultures treated with (a) glutamate (1 mM) and (b) colchicine (1 μM) for 0 min (lane 1), 5 min (lane 2), 30 min (lane 3), 1 h (lane 4), 3 h (lane 5) and 6 h (lane 6). The blots were probed with 8073 or Tau.1, as indicated. Arrows show the positions of the major tau broad band/doublet, and arrowheads indicate the positions of the two intact less phosphorylated and faster-migrating tau species. Numbers to the right of the Figures represent approximate molecular masses (kDa).

Over the course of glutamate treatment, there was a 22% increase in LDH release above control levels after 6 h, but at shorter times up to 3 h, there was no significant increase in LDH release, even though there was already a marked change in tau mobility and immunoreactivity. By 12 h of glutamate treatment there was a near-maximal release of LDH of 94% above control levels. This pattern of LDH release over time during an excitotoxic response is similar to that previously described [27]. We

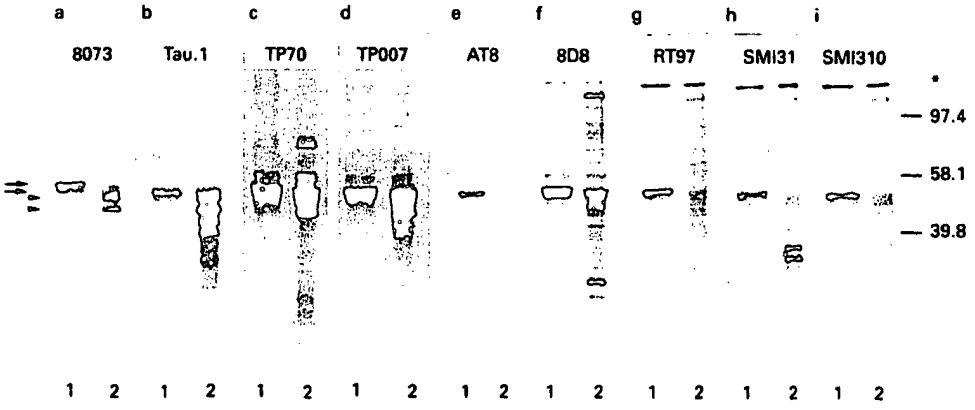

Figure 2 Glutamate-induced dephosphorylation of tau in primary neuronal cultures

Western blots of total cell-lysate proteins from primary 10-day-old rat cortical neuronal cultures treated with glutamate (1 mM) for 0 min (lane 1) or 6 h (lane 2), and incubated with (**a**) 8073, (**b**) Tau.1, (**c**) TP70, (**d**) TP007, (**e**) AT8, (**f**) 8D8, (**g**) RT97, (**h**) SMI31 and (**i**) SMI310, as indicated. Arrows indicate the positions of the major tau broad band/doublet, and arrowheads indicate the positions of the two main less phosphorylated and faster-migrating intact tau species. A minor higher-molecular-mass tau species (approx. 57 kDa) seen with TP70, TP007 and 8D8 is probably a longer tau isoform (see the Results section). Asterisk indicates the higher-molecular-mass band that corresponds to the neurofilament protein NF-H. Numbers to the right of the Figure represent approximate molecular masses (kDa).

also observed a similar pattern of LDH release after colchicine treatment of the primary neuronal cultures (results not shown).

Figure 2 shows Western blots of total cell-lysate protein from 10-day-old cultures that were treated with glutamate (1 mM) for zero (control) or 6 h, and have been probed with a panel of antibodies. TP70 (Figure 2c) and TP007 (Figure 2d) (polyclonal antibodies that recognize the C- and N-termini of tau respectively) both labelled the initial tau broad band/doublet (arrows) and the same strongly immunoreactive faster-migrating species (arrowheads) as recognized by 8073 (Figure 2a) and Tau.1 (Figure 2b) that appear after glutamate treatment of the cultures. On some blots, an additional higher-molecular-mass tau species of approx. 55–57 kDa was recognized by the polyclonal antibodies TP70 and TP007 and the monoclonal neurofilament antibody 8D8. This probably corresponds to a longer tau isoform and can also be detected in 3-day-postnatal rat brain [14]. The labelling of the faster-migrating tau species (arrowheads) by antibodies to both extremities of the tau polypeptide demonstrates that these principal faster-migrating species are not the products of proteolysis, but rather are due to dephosphorylation of tau; these species are also hardly labelled, if at all, by the phosphate-dependent monoclonal antibodies (Figures 2e–2i). Similar dephosphorylation *in vitro* of neuronal tau in cell lysates by alkaline phosphatase in the presence of a cocktail of protease inhibitors also demonstrated that the faster-migrating species were the product of dephosphorylation and not proteolysis (see below and Figure 4). The additional and apparently smaller species than those indicated by arrowheads that were present after glutamate treatment and recognized by Tau.1 (Figure 2b) and TP007 (Figure 2d), are probably tau degradation products, because they were not recognized by the C-terminal antibody TP70 (Figure 2c).

The monoclonal antibodies AT8, 8D8, RT97, SMI31 and SMI310 (Figures 2e–2i respectively), all of which recognize phosphorylated tau epitopes, and which label only the slower-migrating species of the broad band/doublet of tau [14], showed a decrease in intensity of labelling of this tau species after glutamate treatment of cultures for 6 h and, indeed, the labelling by AT8 was abolished. It was with these phosphate-dependent monoclonal antibodies that two differently phosphorylated species of tau were most clearly observed, since in untreated cultures they consistently labelled a narrow and the most slow-migrating fraction of the broad band/doublet of tau (Figures 2e–2i). Conversely, Tau.1 showed an increase in staining intensity of the faster-migrating component of the broad band/doublet after 6 h of glutamate treatment (Figure 2b). These results suggest that a significant proportion of the tau in primary rat cortical cultures is normally highly phosphorylated and becomes dephosphorylated after glutamate treatment. Although neuronal death occurs, since LDH is released, this process is gradual, as has been demonstrated by others [27–30], and the dephosphorylation of tau commences before this lytic stage (Figure 1), but is more evident after 6 h of treatment.

The monoclonal antibodies 8D8, RT97, SMI31 and SMI310 (which are all neurofilament antibodies) all recognized a high-molecular-mass band, corresponding to neurofilament protein NF-H (Figure 2, asterisk), in both control and treated samples. After glutamate stimulation, a second high-molecular-mass band was recognized by all of these neurofilament antibodies that may correspond to neurofilament protein NF-M or a degradation product of NF-H. In addition, these neurofilament monoclonal antibodies variably labelled to different intensities several other species throughout the molecular-mass range that may be degradation products of neurofilaments. On the other hand, as all of these antibodies selectively recognize phosphorylated epitopes, glutamate treatment of the cultures, conversely to its effects on tau, may induce phosphorylation of some proteins that are then cross-reactive with monoclonal antibodies against phosphorylated NF-H. Indeed, 8D8 staining of a band in the NF-M region is strongly increased by glutamate treatment, and since the control sample does not contain a longer species of comparable immunoreactivity, it would appear that this molecular-mass band is most likely due to an increase in neurofilament phosphorylation.

Colchicine-induced dephosphorylation of tau in primary rat cortical neuronal cultures

Colchicine treatment of both 6- and 10-day-old rat primary cortical cultures had a similar effect to glutamate on the

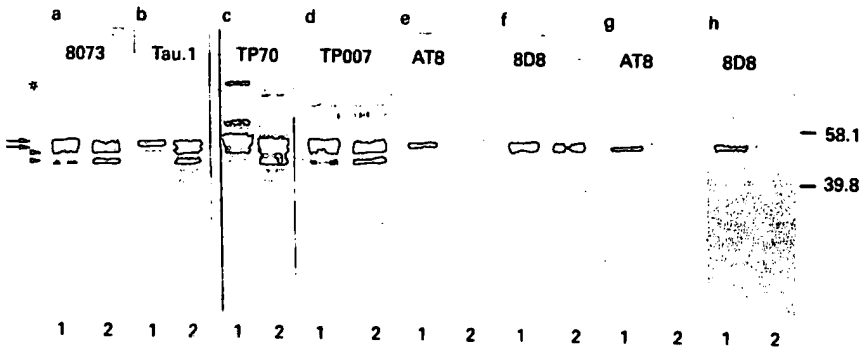

Figure 3 Colchicine-induced dephosphorylation of tau in primary neuronal cultures

(**a**–**f**) Western blots of total cell-lysate proteins from control primary 6-day-old rat cortical cultures (lanes 1) and 6-day-old cultures treated with colchicine (1 μM) for 6 h (lanes 2), and incubated with (**a**) 8073, (**b**) Tau.1, (**c**) TP70, (**d**) TP007, (**e**) AT8 and (**f**) 8D8; (**g**) and (**h**) are Western blots of total cell-lysate proteins extracted from 10-day cultures incubated with (**g**) AT8 and (**h**) 8D8. Arrows indicate the positions of the major tau broad band/doublet, and arrowheads indicate the positions of the two intact less phosphorylated and faster-migrating tau species. As in Figure 2, a minor tau-positive species (approx. 57 kDa) indicates the presence of a longer tau isoform; the presence of this species is most marked with TP70. TP70 probably also labels MAP2C (asterisk), and this too is shifted to faster-migrating forms after colchicine treatment. Numbers to the right of the Figure represent approximate molecular masses (kDa).

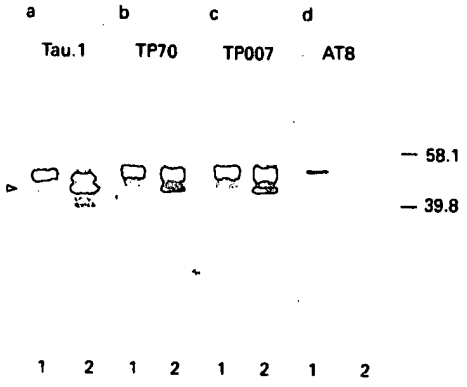

Figure 4 Dephosphorylation *in vitro* of tau extracted from primary neuronal cultures

Western blots of control heat-stable proteins from primary rat cortical cultures (lane 1) and heat-stable proteins from primary cortical cultures treated with alkaline phosphatase for 16 h at 37 °C (lane 2), and probed with (**a**) Tau.1, (**b**) TP70, (**c**) TP007 and (**d**) AT8. Arrowhead indicates the position of the less phosphorylated and faster-migrating tau species. Numbers to the right of the Figure represent approximate molecular masses (kDa).

phosphorylation state of tau, as assessed both by Western-blot analysis (Figure 3) and immunocytochemically (results not shown). The polyclonal antibody 8073 showed the generation of increased amounts of lower-molecular-mass species (arrowheads) on Western blots (Figure 3a), after treatment of 6-day cortical cultures with 1 μM colchicine for 6 h. Tau.1 showed the appearance of two faster-migrating tau species (arrowheads) after colchicine treatment (Figure 3b), whereas AT8 failed to label tau after the same treatment in both 6- and 10-day cultures (Figures 3e and 3g respectively). After treatment with colchicine, the monoclonal anti-neurofilament antibody 8D8 showed a modest decrease in intensity of labelling on 6-day cultures and abolition of labelling of 10-day cultures (Figures 3f and 3h respectively). Again, these changes were not the result of tau proteolytic degradation, since the principal faster-migrating bands (arrowheads) were both labelled by the antibodies TP70 and TP007 (C- and N-terminal anti-tau polyclonal antibodies, respectively) (Figures 3c and 3d respectively), although additional weakly tau-positive bands migrating faster than those indicated by arrowheads probably are degradation products. The higher-molecular-mass band recognized by the polyclonal antibody TP70 (asterisk) is probably due to cross-reactivity with the microtubule-associated protein MAP2C, which is also shifted to a faster-migrating species after colchicine treatment, tau and MAP2C having a high degree of similarity at their C-termini [31]. The higher-molecular-mass tau species also recognized by TP70 (approx. 57 kDa) is referred to above.

Dephosphorylation *in vitro* of tau from primary rat cortical neuronal cultures

In order to confirm further that the observed effects of glutamate and colchicine on tau immunoreactivity were indeed due to dephosphorylation of tau, a heat-stable protein preparation from 10-day primary cortical cultures was treated with alkaline phosphatase in the presence of a cocktail of protease inhibitors (see the Materials and methods section). After enzyme treatment, a proportion of the tau had an increased electrophoretic mobility (Figures 4a–4c, arrowhead) and showed a total loss of labelling with the phosphorylation-dependent antibody AT8 (Figure 4d). This dephosphorylation was similar to, although not identical with, that seen after glutamate and colchicine treatments of the neuronal cultures. Not all of the tau was dephosphorylated by alkaline phosphatase treatment *in vitro*, and we have found it difficult to achieve complete dephosphorylation. The N- and C-terminal antibodies TP70 and TP007 also labelled this dephosphorylated tau (Figures 4b and 4c), demonstrating that this faster-migrating species was the result of dephosphorylation and not degradation of tau. Tau.1 also labelled small amounts of additional faster-migrating species after alkaline phosphatase treatment, which probably were the result of proteolysis (Figure 4a).

Immunocytochemistry of primary rat cortical neuronal cultures

The effects of glutamate and colchicine treatments on the distribution and the phosphorylation status of tau in cultured rat

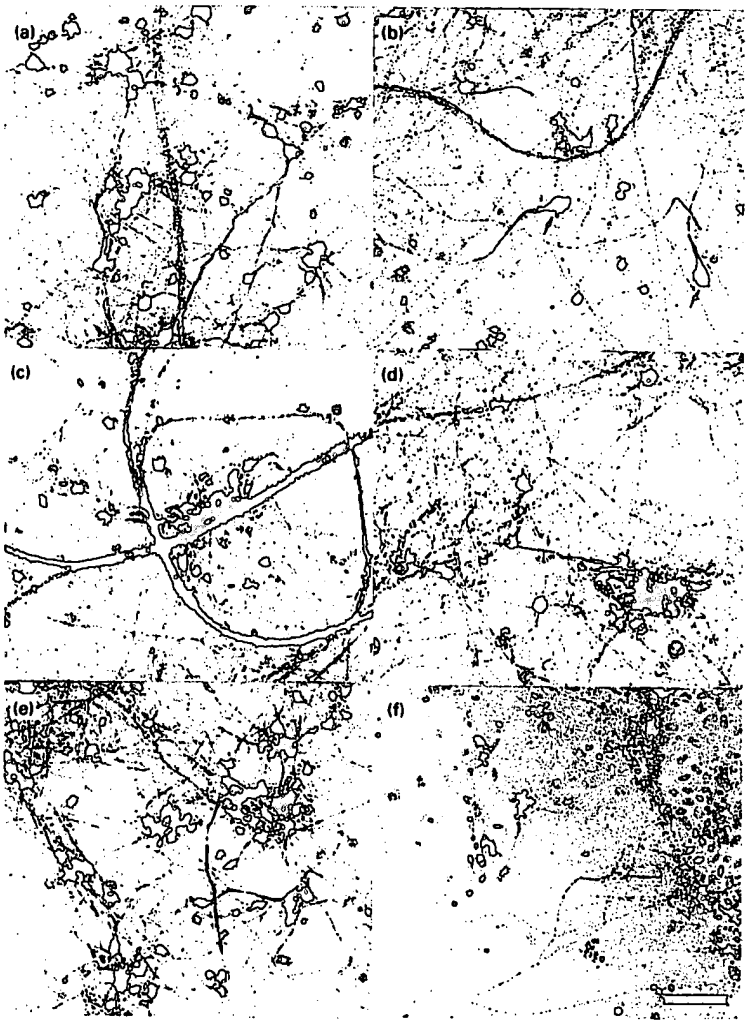

Figure 5 Glutamate-induced dephosphorylation of tau in primary neuronal cultures *in situ*

Immunocytochemical staining of control (**a**, **c** and **e**) and glutamate-treated (1 mM; **b**, **d** and **f**) 10-day-old primary rat cortical neurons with polyclonal anti-tau antibody B19 (**a** and **b**), or monoclonal antibodies Tau.1 (**c** and **d**) and AT8 (**e** and **f**). Glutamate treatment induced an increase in B19 perikaryal immunoreactivity in a subpopulation of neurons (arrows in **b**); many neurons showed an increase in Tau.1 perikaryal immunoreactivity and a decrease in AT8 staining. Scale bar, 50 μm.

cortical neurons was investigated by immunocytochemical labelling *in situ* with polyclonal antiserum B19 and the monoclonal antibodies AT8 and Tau.1.

In 10-day-old control cortical cultures, the polyclonal antibody B19 labelled axons and neuronal cell bodies (Figure 5a); the Tau.1 monoclonal antibody labelled axons with variable intensity, some long axons being strongly labelled, but this antibody labelled the perikarya only weakly (Figure 5c). AT8 displayed labelling very similar to that of B19, i.e. a labelling of cell bodies and axons (Figure 5e). A significant number of neurons also showed a labelling of dendrites or the proximal part of dendrites, with both B19 and AT8 (Figures 5a and 5e). Glutamate treatment (1 mM for 6 h) of 10-day-old cultured cortical neurons induced marked changes in the tau immunoreactivity. The polyclonal B19 antibody still labelled axons and perikarya, but the tau immunoreactivity in perikarya was clearly increased in a significant proportion of neurons (Figure 5b). Tau.1 strongly labelled many neuronal perikarya (Figure 5d), while still demonstrating immunoreactivity with axons. Contrary to this, AT8 immunoreactivity was strongly decreased throughout glutamate-treated neurons (Figure 5f).

Immunocytochemical labelling of 6-day-old control cortical neurons with the polyclonal antibody B19 and monoclonal antibodies Tau.1 and AT8 was similar to that described above for the 10-day-old control cultures (results not shown). In colchicine-treated (1 μM for 6 h) 6-day-old neuronal cultures, the change in immunoreactivity with the polyclonal antibody B19 and monoclonal antibodies Tau.1 and AT8 was similar to that seen after glutamate treatment, in that B19 and Tau.1 both showed an increase in labelling of many neuronal perikarya and AT8 showed a decrease in neuronal immunoreactivity in a significant proportion of neurons (results not shown).

Effect of okadaic acid on glutamate-induced tau dephosphorylation

In order to investigate potential phosphatase involvement in the glutamate-induced dephosphorylation of neuronal tau, we

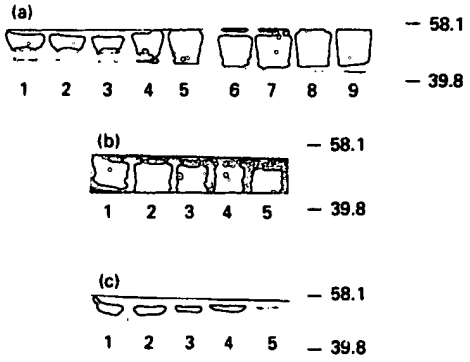

Figure 6 Effects of okadaic acid on glutamate-induced tau dephosphorylation

Western blots of total cell-lysate proteins from untreated 10-day-old rat cortical neuronal cultures (**a**, **b** and **c**, lanes 1) or treated with okadaic acid (250 nM) for 10 min (**a** and **b**, lanes 2), 30 min (**a** and **b**, lanes 3), 1 h (**a** and **b**, lanes 4) and 6 h (**a** and **b**, lanes 5), or okadaic acid (250 nM) and glutamate (1 mM) for 10 min (**a**, lane 6, and **c**, lane 2), 30 min (**a**, lane 7, and **c**, lane 3), 1 h (**a**, lane 8, and **c**, lane 4) and 6 h (**a**, lane 9, and **c**, lane 5) and probed with (**a**) TP70, (**b**) Tau.1 or (**c**) 8D8. Numbers to the right of the Figure represent approximate molecular masses (kDa).

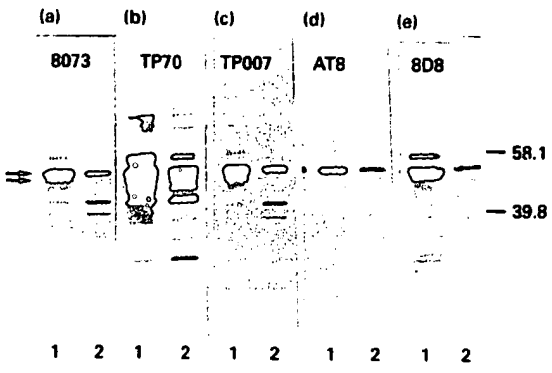

Figure 7 Effects of β_{25-35}-amyloid peptide on primary neuronal cultures

Western blots of heat-stable proteins from 8-day-old control primary rat cortical cultures (lane 1) and cultures treated with aggregated β_{25-35}-amyloid peptide (100 μM) for 24 h (lane 2) and probed with (**a**) 8073, (**b**) TP70, (**c**) TP007, (**d**) AT8 and (**e**) 8D8. Arrows indicate the positions of the major tau broad band/doublet. As in Figure 2, a minor tau-positive species (approx. 57 kDa) indicates the presence of a longer tau isoform; the presence of this species is most marked with TP70 and 8D8. Numbers to the right of the Figure represent approximate molecular masses (kDa).

studied the effects of okadaic acid on this process. However, we found that 250 nM okadaic acid alone further slightly decreased the mobility of tau after 1 h of treatment, as assessed with the polyclonal antibody TP70 (Figure 6a, lane 4), and the intensity of staining of tau by monoclonal antibody Tau.1 after 1 h of okadaic acid treatment was decreased, indicating increased phosphorylation at Ser-199/202 (Figure 6b). Both of these changes are consistent with additional overall phosphorylation of the tau.

When cells were treated with both okadaic acid and glutamate, then the two effects of each agent occurred together. Thus, the two faster-migrating tau species were generated and the slowest-migrating tau band was apparently even further decreased in mobility (Figure 6a, compare lane 1 with lanes 6–9). However, the staining by monoclonal antibody 8D8 was decreased (Figure 6c, lane 5). This implies that the effects of okadaic acid treatment are complex, in that it inhibits some phosphatase activity to cause a further decrease in mobility, but it does not inhibit the phosphatase activity responsible for dephosphorylating the 8D8 epitope in these cultures.

β-amyloid failed to induce PHF-like phosphorylation of tau in primary rat cortical neuronal cultures

Treatment of 8-day-old primary rat cortical neuronal cultures with aggregated β_{25-35}-amyloid peptide (100 μM for 24 h) was associated with marked toxicity (maximal LDH release and histological results not shown). The polyclonal antibodies 8073, TP70 and TP007 all labelled tau extracted from cultures treated with β_{25-35}-amyloid peptide less intensely than control tau (Figures 7a, 7b and 7c, respectively), which is probably due to general protein degradation associated with cell death. These antibodies also recognized faster-migrating immunoreactive species after treatment of the cultures with β_{25-35}-amyloid peptide (Figures 7a, 7b and 7c), some of which are possibly the result of dephosphorylation of tau, as they are labelled by all three polyclonal antibodies, whereas the smaller species are variably recognized and are probably the result of proteolysis. The phosphorylation-dependent monoclonal antibodies AT8 and 8D8 also did not recognize these faster-migrating bands, but labelled the initial broad tau band/doublet less intensely after β_{25-35}-amyloid peptide treatment (Figures 7d and 7e respectively) of the cultures. However, this decrease in immunoreactivity with the phosphate-dependent antibodies was not obviously greater than that observed with the polyclonal antibodies, demonstrating that tau degradation rather than dephosphorylation is the predominant response to treatment with β_{25-35}-amyloid peptide.

We have tried many different experimental paradigms in order to investigate the effect of β_{25-35}-amyloid on tau phosphorylation in primary neuronal cultures, and the results shown in Figure 7 are typical of the data obtained. These include low- and high-density hippocampal and cortical cultures which have been grown for up to 31 days and treated both acutely and chronically with various concentrations of peptide, in either the presence or the absence of glutamate. Although we have consistently been able to induce toxicity, we have never seen any evidence of an increase in tau phosphorylation, such as might be indicative of PHF-like changes in the neuronal cytoskeleton.

DISCUSSION

We and others have demonstrated that a proportion of foetal tau in rat and human brain is in an elevated state of phosphorylation compared with adult brain tau. Thus, it has been shown by Western-blot analysis, using antibodies that discriminate between phosphorylated and non-phosphorylated epitopes, that foetal tau is a mixture of at least two major species, one of which is more highly phosphorylated than the other [14–17]. Here we have shown that tau extracted from primary cultures of rat cortical neurons appears to be very similar to that extracted directly from foetal brain, and these two phosphorylation states of tau persist in the cultured neurons for at least 10 days.

In the present study we have demonstrated that treatment of primary neuronal cultures with glutamate or colchicine results in dephosphorylation of the more heavily phosphorylated tau species. This dephosphorylation is both progressive and a phenomenon that occurs during the process of neuronal degeneration, but before significant cell lysis has occurred. One laboratory has reported that glutamate treatment of neurones in

culture induces an increase in tau phosphorylation [32,33], but these studies were over short periods of treatment, and we have been unable to confirm these findings, and the reason for this discrepancy is as yet unclear.

Although the faster-migrating tau component of the broad band/doublet present initially in the cultured neurons, and which is labelled by Tau.1 but not the phosphate-dependent monoclonal antibodies, is clearly less phosphorylated than the slightly slower migrating species, it nevertheless is phosphorylated, since treatment of the cultures with glutamate resulted in the appearance of additional and faster-migrating species (arrowheads in Figures 1–3). This was confirmed by the treatment *in vitro* with alkaline phosphatase of heat-stable tau extracted from the cultures. It is therefore likely that this faster-migrating species of foetal tau is phosphorylated at sites other than those recognized by the panel of available monoclonal antibodies, all of which recognize phosphorylated serine/threonine residues followed by proline, i.e. SP or TP motifs. These extra phosphorylation sites may reside in the C-terminal half of the molecule, since we and others have shown that phosphorylation *in vitro* of the C-terminal half of recombinant tau by cyclic AMP-dependent protein kinase or Ca^{2+}/calmodulin-dependent protein kinase, probably at sites other than SP or TP residues, results in a significant upward shift in mobility of tau [34–39].

The dephosphorylation of foetal tau induced by glutamate or colchicine was incomplete, because not all the tau shifted to the fastest-migrating form. Nevertheless, the epitopes recognized by the phosphate-dependent monoclonal antibodies were in some experiments completely dephosphorylated. The mechanism by which glutamate and colchicine induce a dephosphorylation of tau in primary neuronal cultures has yet to be elucidated, but, since both these agents give rise to an increase in perikaryal tau immunoreactivity, it is possible that they share a similar mechanism that might involve a decrease in the number of polymerized microtubules. Glutamate could act by increasing intracellular Ca^{2+} levels, thus leading to microtubule destabilization and consequently the release of tubulin-bound tau [3,32,40]. Direct destabilization of microtubules by colchicine, independently of Ca^{2+} influx, might have the same effect. Phosphorylation appears to regulate the tau–microtubule interaction [41–44], and so, if there is a shift from polymerized to unpolymerized tubulin, then dephosphorylation of tau may be a compensatory mechanism to promote microtubule assembly after neurotoxic insult. Sygowski et al. [45] also demonstrated that colchicine treatment of NIH 3T3 fibroblasts expressing transfected tau resulted in the tau becoming less phosphorylated, although not all microtubule-destabilizing agents produced this effect.

The regulation of tau phosphorylation is probably complex, since experiments with okadaic acid treatment of neuronal cultures produced a slight decrease in electrophoretic mobility of tau, and this was still observed when the cultures were treated with a mixture of glutamate and okadaic acid, although under the latter conditions staining by the monoclonal antibody 8D8 was decreased markedly. This implies that inhibition of protein phosphatases PP1 and PP2A by okadaic acid causes an electrophoretic-mobility shift, but that these phosphatases do not act on the 8D8 epitope. Indeed, studies *in vitro* have demonstrated that protein phosphatases PP1, PP2A, PP2B and PP2C can all dephosphorylate tau, but with differential potencies towards individual sites [46–48].

Our immunocytochemical results confirm the findings of Mattson and colleagues [3,40] that both glutamate and colchicine treatment of neurons in primary culture results in increased perikaryal immunoreactivity of tau, but our data also show that this relocation of tau from axons to neuronal cell bodies is accompanied by a decrease in phosphorylation of the protein, consistent with the Western blot analysis (Figures 1, 2 and 3). These immunocytochemical changes in the presence of colchicine and glutamate occurred during the course of neuronal degeneration, but were apparently not the consequence of cell death.

This increase in perikaryal tau immunoreactivity in primary neurons treated with glutamate, colchicine or glutamate, in conjunction with β-amyloid peptides, has previously been suggested to mimic changes observed in neurons containing PHF in Alzheimer's disease, and it was proposed that one mechanism by which β-amyloid is neurotoxic is by acting synergistically with glutamate [2,49,50]. However, the dephosphorylation of tau observed in glutamate- and colchicine-treated primary neurons in the present study is opposite to the increase in phosphorylation that is associated with the conversion of adult brain tau into PHF-tau. We found, however, that β_{25-35}-amyloid alone induced no obvious change in the phosphorylation state of tau in primary neuronal cultures, and we failed to observe any synergistic effect of β_{25-35}-amyloid and glutamate on tau phosphorylation. Using the available antibodies, on no occasion did we observe an increase in the proportion of tau in an elevated state of phosphorylation after β_{25-35}-amyloid treatment of neuronal cultures, even when β_{25-35}-amyloid-induced toxicity was clearly apparent.

In conclusion, although the immunocytochemical changes seen in primary neurons treated with glutamate and colchicine superficially mimic those seen in neurons containing PHF in Alzheimer's disease, i.e. an accumulation of tau in neuronal perikarya [51], this redistribution is associated with a dephosphorylation rather than increased phosphorylation of tau. This dephosphorylation is associated with neuronal degeneration (as assessed by release of LDH), but commences before there is any significant cell lysis. The pattern of phosphorylation changes is thus opposite to that associated with PHF formation in Alzheimer's disease. However, since primary rat cortical neurons do contain a significant proportion of tau in an elevated state of phosphorylation, they thus represent a valuable cellular model for investigating those mechanisms that regulate tau phosphorylation during development, and for identifying candidate phosphatases or kinases that may, nevertheless, be involved in tau phosphorylation in the pathogenesis of Alzheimer's disease.

We thank Dr. Jim Cohen for advice on preparing primary neuronal cultures and Innogenetics for providing the AT8 antibody. This work was supported by the British Medical Research Council, the Wellcome Trust, Glaxo Research and Development Ltd., the British Council, NATO, the Belgian F.R.S.H. (3.450.91) and the Fonds de Recherché Divvy.

REFERENCES

1 Smith, C. and Anderton, B. H. (1955) Neuropathol. Appl. Neurobiol. **12**, 376–389
2 Mattson, M. P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I. and Rydel, R. E. (1992) J. Neurosci. **12**, 376–389
3 Mattson, M. P. (1992) Brain Res. **592**, 107–118
4 Brion, J. P., Couck, A. M., Passareiro, E. and Flament-Durand, J. (1985) J. Submicrosc. Cytol. **17**, 89–96
5 Wischik, C. M., Novak, M., Edwards, P. C., Klug, A. Tichelaar, W. and Crowther, R. A. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 4884–4888
6 Kosik, K. S., Orecchio, L. D., Binder, L., Trojanowski, J. Q., Lee, V. M. and Lee, G. (1988) Neuron **1**, 817–825
7 Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D. and Crowther, R. A. (1989) Neuron **3**, 519–526
8 Brion, J. P., Hanger, D. P., Couck, A. M. and Anderton, B. H. (1991) Biochem. J. **279**, 831–836
9 Hanger, D. P., Brion, J. P., Gallo, J. M., Cairns, N. J., Luthert, P. J. and Anderton, B. H. (1991) Biochem. J. **275**, 99–104

10 Goedert, M., Spillantini, M. G., Cairns, N. J. and Crowther, R. A. (1992) Neuron **8**, 159–168
11 Greenberg, S. G., Davies, P., Schein, J. D. and Binder, L. I. (1992) J. Biol. Chem. **267**, 564–569
12 Brion, J. P., Couck, A. M., Robertson, J., Loviny, T. L. and Anderton B. H. (1993) J. Neurochem. **60**, 1372–1382
13 Lichtenberg-Kraag, B., Mandelkow, E. M., Biernat, J., Steiner, B., Schroter, C., Gustke, N., Meyer, H. E. and Mandelkow, E. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 5384–5388
14 Brion, J. P., Smith, C., Couck, A. M., Gallo, J. M. and Anderton, B. H. (1993) J. Neurochem. **61**, 2071–2080
15 Kenessey, A. and Yen, S. H. (1993) Brain Res. **629**, 40–46
16 Watanabe, A., Hasegawa, M., Suzuki, M., Takio, K., Morishima-Kawashima, M., Titani, K., Arai, T., Kosik, K. S. and Ihara, Y. (1993) J. Biol. Chem. **268**, 25712–25717
17 Kanemaru, K. and Ihara, Y. (1991) Tanpakushitsu Kakusan Koso **36**, 2–11
18 Brambletl, G. T., Goedert, M., Jakes, R., Merrick, S. E., Trojanowski, J. Q. and Lee, V. M. (1993) Neuron **10**, 1089–1099
19 Gallo, J. M., Hanger, D. P., Twist, E. C., Kosik, K. S. and Anderton, B. H. (1992) Biochem. J. **286**, 399–404
20 Binder, L. I., Frankfurter, A. and Rebhun, L. I. (1985) J. Cell Biol. **101**, 1371–1378
21 Mercken, M., Vandermeeren, M., Lubke, U., Six, J., Boons, J., van de Voorde, A., Martin, J. J. and Gheuens, J. (1992) Acta Neuropathol. **84**, 265–272
22 Sternberger, N. H., Sternberger, L. A. and Ulrich, J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4274–4276
23 Coleman, M. P. and Anderton, B. H. (1990) J. Neurochem. **54**, 1548–1555
24 Hanger, D. P., Hughes, K., Woodgett, J. R., Brion, J. P. and Anderton, B. H. (1992) Neurosci. Lett. **147**, 58–62
25 Koh, J. Y. and Choi, D. W. (1987) J. Neurosci. Methods **20**, 83–90
26 Laemmli, U. K. (1995) Nature (London) **227**, 680–685
27 Dawson, V. L., Dawson, T. M., Bartley, D. A., Uhl, G. R. and Snyder, S. H. (1993) J. Neurosci. **13**, 2651–2661
28 Choi, D. W. (1988) Neuron **1**, 623–634
29 Koh, J. Y., Goldberg, M. P., Hartley, D. M. and Choi, D. W. (1990) J. Neurosci. **10**, 693–705
30 Choi, D. W. (1987) J. Neurosci. **7**, 369–379
31 Kosik, K. S., Orecchio, L. D., Bakalis, S., Duffy, L. and Neve, R. L. (1988) J. Neurochem. **51**, 587–598
32 Sindou, P., Couratier, P., Barthe, D. and Hugon, J. (1992) Brain Res. **572**, 242–246
33 Sautiere, P. E., Sindou, P., Couratier, P., Hugon, J., Wattez, A. and Delacourte, A. (1992) Neurosci. Lett. **140**, 206–210
34 Baudier, J. and Cole, R. D. (1987) J. Biol. Chem. **262**, 17577–17583
35 Steiner, B., Mandelkow, E. M., Biernat, J. et al. (1990) EMBO J. **9**, 3539–3544
36 Kojima, M., Takahashi, N., Ikeuchi, T. and Hatanaka, H. (1992) Mol. Brain Res. **16**, 267–273
37 Robertson, J., Loviny, T. L., Goedert, M., Jakes, R., Murray, K. J., Anderton, B. H. and Hanger, D. P. (1993) Dementia **4**, 256–263
38 Correas, I., Diaz-Nido, J. and Avila, J. (1992) J. Biol. Chem. **267**, 15721–15728
39 Johnson, G. V. (1992) J. Neurochem. **59**, 2056–2062
40 Mattson, M. P. (1990) Neuron **4**, 105–117
41 Scott, C. W., Spreen, R. C., Herman, J. L., Chow, F. P., Davison, M. D., Young, J. and Caputo, C. B. (1993) J. Biol. Chem. **268**, 1166–1173
42 Scott, C. W., Vulliet, P. R. and Caputo, C. B. (1993) Brain Res. **611**, 237–242
43 Lindwall, G. and Cole, R. D. (1984) J. Biol. Chem. **259**, 5301–5305
44 Drechsel, D. N., Hyman, A. A., Cobb, M. H. and Kirschner, M. W. (1992) Mol. Biol. Cell **3**, 1141–1154
45 Sygowski, L. A., Fieles, A. W., Lo, M. M., Scott, C. W. and Caputo, C. B. (1993) Mol. Brain Res. **20**, 221–228
46 Gong, C. X., Grundke-Iqbal, I., Damuni, Z. and Iqbal, K. (1994) FEBS Lett. **341**, 94–98
47 Gong, C.-X., Grundke-Iqbal, I. and Iqbal, K. (1994) Neuroscience **61**, 765–772
48 Drewes, G., Mandelkow, E. M., Baumann, K., Goris, J., Merlevede, W. and Mandelkow, E. (1993) FEBS Lett. **336**, 425–432
49 Koh, J. Y., Yang, L. L. and Cotman, C. W. (1990) Brain Res. **533**, 315–320
50 Sandhu, F. A., Porter, R. H., Eller, R. V., Zain, S. B., Salim, M. and Greenamyre, J. T. (1993) J. Neurochem. **61**, 2286–2289
51 Bancher, C., Brunner, C., Lassmann, H., Budka, H., Jellinger, K., Wiche, G., Seitelberger, F., Grundke-Iqbal, I., Iqbal, K. and Wisniewski, H. M. (1989) Brain Res. **477**, 90–99

Received 31 October 1994/29 March 1995; accepted 5 April 1995